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Polyhydroxyalkanoates (PHAs) production from agro-industrial and urban wastes, CO_2 and energy crops by mixed microbial cultures

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Summary

1. Introduction 1
1.1 Overview: circular economy and biorefineries 1
1.2 Feedstocks for biorefineries 2
Organic waste streams: cheese whey and the OFMSW 3
Non-food energy crops: *Arundo donax* 6
Carbon dioxide 7
1.3 Bioplastic and Polyhydroxyalkanoates (PHAs) 9
PHAs 9
PHAs applications 12
PHAs production by pure microbial cultures 12
PHAs production by mixed microbial cultures (MMCs) 13
PHAs biosynthesis 15
PHAs production by MMCs from complex carbon streams 16
PHAs extraction from MMCs 19
2. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture 22
2.1 Introduction 22
2.2 Materials and methods 24
2.3 Results and Discussion 29
2.4 Conclusions 37
3. Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture 39
3.1 Background 40
3.2 Methods 41
3.3 Results 49
3.4 Discussion 54
3.5 Conclusions 59
4. Bioconversion of giant cane for integrated production of biohydrogen, carboxylic acids and polyhydroxyalkanoates (PHAs) in a multistage biorefinery approach 61
4.1 Introduction 61
4.2 Experimental section 63
4.3 Results and Discussion 69
4.4 Mass balance and economic evaluations 77
5. Bio-electrorecycling of carbon dioxide into bioplastics 82
5.1 Introduction 82
5.2 Materials and methods 84

5.3 Results and discussion 89
5.4 Conclusions 96
6. Non-ionic surfactants as a pretreatment to optimize PHA extraction from mixed microbial cultures by using DMC 97
6.1 Introduction 97
6.2 Materials and methods 99
6.3 Results and discussion 101
6.4 Conclusions 106
7. Conclusions 107

Preface

The following PhD thesis is about polyhydroxyalkanoates (PHAs) production from mixed microbial cultures (MMCs) by using different renewable carbon sources as substrates and it is organized in seven chapters.

Chapter 1 explains the context of the work, namely the concept of circular economy and biorefinery, introducing the possibility to integrate polyhydroxyalkanoates-PHAs production into a biorefinery approach starting from different renewable and low cost carbon sources. After that, PHAs and their production from MMCs have been explored.

Chapter 2 is represented by a published work about PHAs production from fermented cheese whey by using a MMC.

Chapter 3 is represented by a published work where PHAs production from a MMC is performed by using percolates of the organic fraction of municipal solid waste (OFMSW) as carbon source.

Chapter 4 is represented by a submitted work where PHAs production from a MMC is integrated into a biorefinery approach starting from the non-food crop *Arundo donax* L.

Chapter 5 is represented by a published work on PHAs production from a MMC by using as substrate a mixture of organic acids produced via microbial electrosynthesis (MES) from CO_2.

Chapter 6 is about the development of a novel strategy to extract PHAs from MMCs by adopting non-ionic surfactants and dimethyl carbonate as green extracting agents.

Chapter 7 is represented by the conclusions of this work.

1. Introduction

1.1 Overview: circular economy and biorefineries

Modern society is forced to deal with two main problems:

- a growing scarcity of resources from which derives, particularly in Europe (where the highest per capita consumption of raw materials in the world is recorded), a growing dependence on the importation of fossil origin raw materials;[1]
- a huge amount of waste, mainly produced along some supply chains (first of all the food sector), which requires costly disposal procedures, both from an economic and environmental point of view.[2]

The unsustainability of this model originated the concept of "circular economy" that aims to reduce the environmental impact through measures in favour of a more sustainable development, such as the use of renewable energy and waste recycling.

"Closing the loop" has become the watchword for building a resilient, resource efficient and low-carbon society. It is the path from which the European economy must pass to become sustainable and competitive, it's a challenge for the entire world, which can no longer afford the thoughtless use of resources and an unsustainable system of production and consumption. The concept of circular economy is based on six business actions: regenerate, share, optimise, loop, virtualise, exchange; it is mainly focused on stock optimization through three major loops: reuse and re-marketing for goods, product-life extensions for goods and a recycling loop for molecules (secondary resources).[3]

Figure 1.1 shows a scheme of the circular economy model.

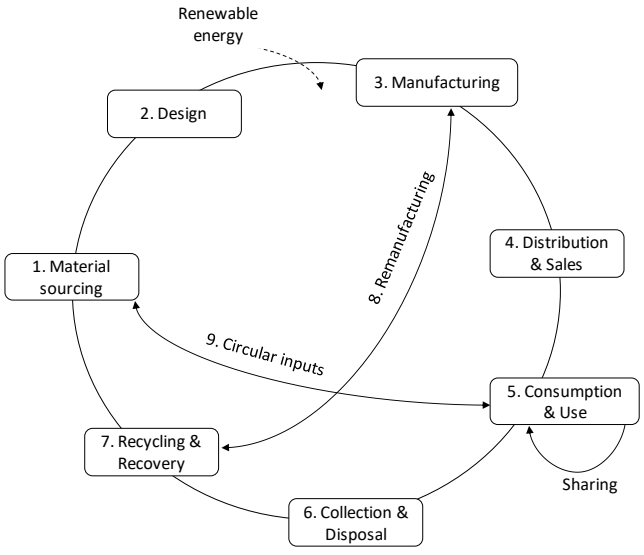

Figure 1.1 *Resource flow through a value chain in a circular economy, elaborated from Kalmykova et al.* [3]

Unlike the traditional linear model "make, use, dispose", the circular economy keeps resources in use for as long as possible, extracts the maximum value from them whilst in use, then recovers and regenerates products and materials at the end of each service life.[4]

The basis of the circular economy could be represented by the biorefinery. The most commonly accepted definition for the term biorefinery is the one introduced by the International Energy Agency (IEA) in Task 42 on bioenergy, according to which it represents a productive system aimed at the sustainable conversion of biomass into a variety of marketable bio-based products and bioenergy.[5]

The current classification system for biorefineries is based on four features through which it is possible to identify and describe them: feedstocks, conversion processes, platforms and products.[6]

Biorefineries feedstocks can be collected under the general term of biomass, which, according to the European Directive 2009/28/EC on the promotion of energy from renewable sources, includes the biodegradable fraction of products, waste and residues from agriculture, forestry and related industries, as well as the biodegradable fraction of urban and industrial waste [7].

Biorefineries conversion processes can be biochemical (i.e. fermentation and enzymatic conversion), thermochemical (i.e. pyrolysis and gasification), chemical (i.e. acid hydrolysis, synthesis and esterification) and mechanical (i.e. pressure and fractionation). In biorefineries, platforms (i.e. biogas, syngas and C5/C6 sugars) represent those intermediate products capable of connecting different types of biorefineries and their processes, whose number is an indicator of the system complexity. The main products obtainable from biorefineries are energy (i.e. liquid and gaseous fuels, such as bioethanol, biodiesel and biogas) and materials (i.e. chemicals, polymers, food and animal feed and additives for cosmetics and medicines).[6,7]

1.2 Feedstocks for biorefineries

Agro-food industry by-products and food waste result of great relevance for biorefinery purposes; nowadays they are only partially valorized at different added-value levels (spread on land, animal feed, composting, or anaerobic digestion and incineration), being mainly disposed in landfill, with significant costs and negative impacts on the sustainability of the agro-food processing industry.[2] On the other side, suitable feedstocks for biorefineries can be also represented by non-food energy crops, by integrating high added-value bioproducts production into efficient biofuel production processes, thus valorizing each component of the starting lignocellulosic material.[8]

The integrated valorisation of such organic streams with the production of added-value fine chemicals, materials, biofuels and water is a new and challenging development (Fig. 1.2).[2,9]

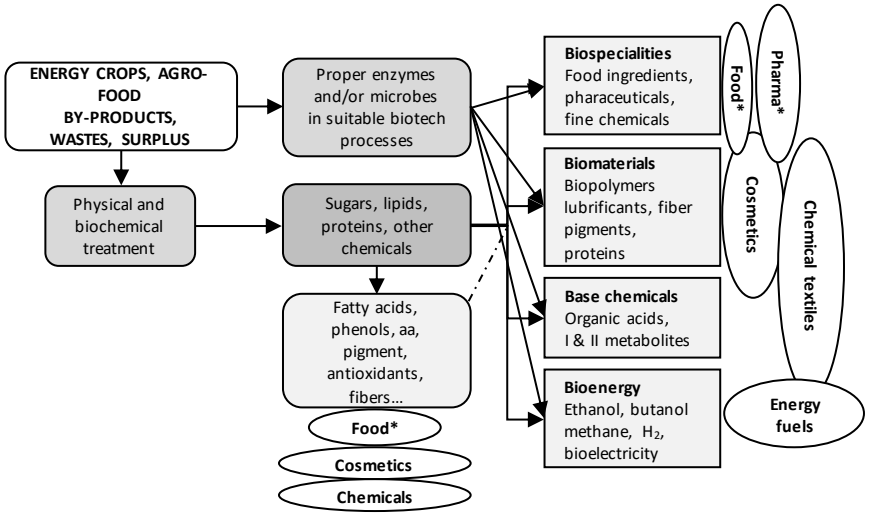

Figure 1.2 *Main features of a biorefinery based on biological processing (*normally not feasible when waste is the feedstock), elaborated from Fava et al.* [2]

In particular, in this PhD thesis the production of biopolymers polyhydroxyalkanoates-PHAs was integrated as a step into simple or more complex biorefinery approaches in which cheese whey, the organic fraction of municipal solid waste (OFMSW), *Arundo donax* and CO_2 were used as feedstocks.

Organic waste streams: cheese whey and the OFMSW

Concerning the dairy industry, cheese whey represents its main by-product, globally produced in the amount of 120 million tons per year, 66 % of which generated by EU.[10] Cheese whey is a green-yellowish liquid, resulting from the precipitation and removal of milk casein in cheese making process. It has a high carbon load (COD= 50-102 g L^{-1}) and it is composed by lactose (39-60 g L^{-1}; about 90 % of the organic load), fat and proteins (0.99-10.58 g L^{-1} and 27-60 g L^{-1}, respectively) and mineral salts (4.6-8 g L^{-1}), mainly represented by NaCl, KCl and calcium phosphates.[11]

In Lombardy Region (Northern Italy) about 3.5 million tons of cheese whey (data of 2015) are produced yearly, that is about 37 % of the total cheese whey produced in Italy and the 2.91 % of the global production.[12] This huge amount of cheese whey is only partly used (about 50 %) directly as animal feed or to produce powder for feed and food [13] and Ricotta cheese, being the remaining part not traceable or cleaned [14]. To minimize the amount of cheese whey annually

disposed, different strategies have been developed, aimed to the production of energy (biogas and biofuel) and goods with high benefits (organic acids, exopolysaccharides, amino acids, vitamins, biopolymers, antibiotics, enzymes and single cell proteins) (Table 1.1).[15] These processes are able to transform cheese whey in products of interest and at the same time to generate low carbon load effluents, thus reducing its environmental impact.[11]

Table 1.1 *Examples of cheese whey biological and physico-chemical treatments* [11]

Treatment	**COD_{in}** ($kg\ m^{-3}$)	**Removal** (%)	**Products**	**By-products**	**Effluent** ($kg\ m^{-3}$)
			Biological		
Aerobic digestion	0.55-70	COD= 53-99	-	Excess biomass sludge Effluent	COD= 0.04-24
Anaerobic digestion	0.5-79	COD= 36-99	Gas (53-79 % of CH_4)	Excess biomass sludge Effluent	COD= 0.1-33
Ferm. to ethanol	35-200 as sugar	Sugar= 33-100	Ethanol (2.1-81 $kg\ m^{-3}$)	Excess biomass sludge Effluent	Sugar= 0-48
Ferm. to hydrogen	10-89	Sugar= 86-97	H_2 (20-88 %)	Excess biomass sludge Effluent	Sugar= 0.1-6.1 COD= 4-28
Ferm. to lactic acid	30-100 as sugar	Sugar= 34-100	Lactic acid (3.8-52 $kg\ m^{-3}$)	Excess biomass sludge Effluent	Sugar= 0-32
Microbial Fuel Cell	0.35-96.5	COD= 5-100	Electricity (18.4-16700 $mW\ m^{-2}$)	Excess biomass sludge Effluent	COD= 0.02-91.7
			Physico-chemical		
Precipitation	-	Proteins= 62-87	Proteins	Precipitate Supernatant	-
Coagulation/ Flocculation	-	Fats= 70-80 Proteins= 62-85 Lactose= 4.5-9.1	Proteins	Precipitate Supernatant	Lactose= 42
Electrochemical flocculation	27.6	COD= 53 Proteins= 74	Proteins	Precipitate Supernatant	COD= 13
Microfiltration	-	Fats= 99 Proteins= 28-85 Lactose= 6.5-19.5	Protein concentrate	Concentrate Permeate	Fats= 0.08-0.6 Lactose= 31-47 Proteins= 2-6
Ultrafiltration	100	COD= 43 Fats= 100 Lactose= 3.2-40 Proteins= 56-81	Protein and lactose concentrates	Concentrate Permeate	COD= 54 Fats= 0 Lactose= 20-56 Proteins= 0.2-2.5
Nanofiltration	53-100	COD= 74-98 Lactose= 89-99.8 Proteins= 87-100	Lactose and protein concentrates	Concentrate Permeate	-
Reverse Osmosis	44-100	COD= 90-94 Proteins= 94-96	Protein and lactose concentrates	Concentrate Permeate	COD= 2.5-7

The term food waste (FW) is referred to the organic material produced for human consumption and discarded, lost or degraded, primarily at manufacturing, retail and consumption stages.[16] In Europe, almost 87.6 million tons of FW are produced annually.[17] These wastes are characterized by both high moisture and high biodegradability, thus creating adverse environmental impacts if landfilled (odours, fires, volatile organic compounds, groundwater contamination by leachate, global climate changes, etc.) and also high disposal costs (75-120 € ton^{-1}) [16]. European

and national legislation have focused on avoiding FW landfilling by treating it through a thermal process (incineration) or, more frequently, by biological processes (anaerobic digestion and composting) to be carried out on the separately collected organic fraction of municipal solid waste (OFMSW).[18] Despite the high biological value of FW, traditional management solutions do not consider it as a precious resource, adding only a small amount of value to the final product, i.e. 50-130 € ton^{-1} of biomass for electricity production and 4-8 € ton^{-1} of biomass for compost production.[19]

FW is an organic matrix rich in valuable molecules such as starch, cellulose, hemicellulose, lignin, proteins, lipids and organic acids that could be managed in a more sustainably economic way by using it as a raw material for bulk chemicals production.[19] Many studies reported the use of FW for the production of high added value molecules such as organic acids, single cell oils, enzymes and polymers (Table 1.2),[20] an approach which is more financially rewarding in comparison with compost and biomethane production (850 € ton^{-1} of biomass for bulk chemicals production from FW) [21].

Table 1.2 *Examples of FW biological treatments for biofuels and high added-value productions*

Product	**Microorganism / enzyme**	**Pre treatment**	**Yield (on waste)**	**Yield (on sugar)**	**Ref.**
Biogas	Codigestion with primary sludge and thickened excess activated sludge in two-stage quasi-continuous mode	-	532 (mL g^{-1} VS)	-	[22]
Biohydrogen	Ferm. to H_2 with anaerobic mixed microbial culture	Ultrasonication	149 (mL g^{-1} VS)	-	[22]
Bioethanol	α-Amylase, glucoamylase, and protease	-	0.36 (g g^{-1} dry matter)	-	[22]
Lactic acid	*L. rhamnosus* KY-3	Propionic fermentation, glucoamylase	0.55 (g g^{-1})	0.84 (g g^{-1})	[20]
Citric acid	*A. niger* DS1	Dried, ground	0.11 (g g^{-1})	0.47 (g g^{-1})	[20]
Succinic acid	Rec. *E.coli*	Fermentation with *A. awamori* and *A. oryzae*	0.22 (g g^{-1})	0.56 (g g^{-1})	[20]
Single cell oils	*Cunninghamella echinulata* CCF-103	H_2SO_4 treatment, autoclave, filtration, pH adjustment to 6	-	0.25 (g g^{-1})	[20]
ABE (Acetone Butanol Ethanol)	*Clostridium beijerinckii* P260	-	-	0.38 (g g^{-1})	[23]
Mix carbohydrases	*A. niger* CJ-5	-	33,310 (U enzyme g^{-1} dry matter)	-	[24]

Since biorefineries are able to treat all of the organic streams above mentioned and, considering that the European Union estimates report a high production of biodegradable waste, projected to increase on average by 10 % by 2020 [25], the potential consequences derived from the

application of biorefineries in the organic waste and by-products management are evident both in terms of industrial development and creation of new job opportunities, both for the contribution to the reduction of dependence on fossil energy sources and natural resources [7].

Non-food energy crops: *Arundo donax*

During the last few years, lignocellulosic materials have started to be considered as promising feedstocks for biorefineries due to their considerable potential to be converted into a wide variety of industrially relevant bioproducts.[26] Lignocellulosic biomasses, which refer to crops, crops residues and forestry biomass, have a complex and heterogeneous structure and composition, mainly represented by the carbohydrates cellulose and hemicellulose, and lignin.[8] The high cellulose and hemicellulose content is a potential source of sugar (around 70 %), in this regard, a key step in biorefineries developed from lignocellulosic feedstocks is represented by the fractionation of lignocellulose into its major components, followed by sugars separations, which can be achieved by adopting several pre treatments: physical (i.e. grinding and milling), chemical (i.e. acids or ionic liquids), physicochemical (i.e. steam, hot water or ammonia fibre expansion) and biological (i.e. enzymes and fungi).[26]

Once the sugars are released from the complex structure of lignocellulose, it is possible to use them for the production of bio-based products, such as composites, fine chemicals, biofuels and enzymes.[8,26]

Among the possible non-food energy crops, in this PhD thesis the attention has been focused on *Arundo donax*. *A. donax* is a perennial herbaceous plant widespread in different environments, able to grow on several kind of soils;[27] its main feature is represented by the huge amount of biomass achieved per unit of cultivated surface area, which is significantly higher than that obtainable from other traditional crops: an average production of 60-70 tons of total solids per Ha was recently obtained in an experimental field in northern Italy.[28,29] Moreover, *A. donax* cultivation involves limited investment and low maintenance costs, due to the low agronomic interventions, low water and fertilizer requirement and no phytosanitary product applications.[29] All of the characteristics above mentioned, together with the lack of competition with food production,[30] have made *A. donax* a valuable candidate for the developing of an economically sustainable biorefinery for the production of biofuel and high added-value bioproducts [31,32] (Table 1.3).

The cellulose and hemicellulose content in *A. donax*, coupled with the high crop yield, represents a significant potential source of lignin complex, hexoses (C6, i.e. glucose) and

pentoses (C5, i.e. xylose and arabinose),[33] which are all interesting platform chemicals with many industrial application [34].

Table 1.3 *Examples of biofuels and bioproducts productions by using A. donax lignocellulosic biomass as feedstock*

Product	Treatment	Yield	Ref.
Biomethane	Anaerobic digestion	9,518 (Nm^3 CH_4 Ha^{-1})	[28]
Biohydrogen	Dark fermentation	3 (mol H_2 mol^{-1} glucose)	[35]
Xylo-oligosaccharides	Isothermal autohydrolysis	17.7 (g 100 g^{-1} dry raw material)	[36]
γ-valerolactone (GVL)	Acid hydrothermal conversion, hydrogenation	16.6 (g 100 g^{-1} dry raw material)	[37]
Lignin; hemicellulose; cellulose nanocrystals	Acidic leaching, alkaline treatment and concentrated sulphuric acid hydrolysis	13; 15; 11 (g 100 g^{-1} dry raw material)	[38]
Levulinic acid	Hydrothermal treatment, acid-catalysed	20-23 (g 100 g^{-1} dry raw material)	[34]

Carbon dioxide

CO_2 release in the atmosphere, which is one of the main contributor of the global warming, is the product of fossil fuel combustion and of many industrial activities. CO_2 is indispensable for photosynthetic organisms, which represent a natural system for its consumption, while its use as substrate for industrial productions has been receiving great interest although limited by the high costs needed for its capture and storage.[39] Nowadays, the possibility to use CO_2 as a building block for chemicals and materials production is considered both as a way to reduce its accumulation in the atmosphere and to partially substitute non renewable fossil sources. This interest is largely driven by CO_2 features that make it an ideal feedstock in a biorefinery context. In facts CO_2 is a safe, inert and renewable carbon source, moreover it is not expensive, it's abundant and well distributed all over the world.[39,40] Biological CO_2 sequestration methods using microorganisms as catalysts could lead to important advances compared to conventional CO_2 capture methods, which are usually considered expensive.[41] In this sense, in this PhD thesis a work related to bioplastic-PHAs production from a mixture of organic acids was performed by using the organic acids electrosynthesized by microorganisms (MES) from CO_2.
In table 1.4 the state of the art of CO_2 utilization is reported.

Table 1.4 *Main applications of CO_2, elaborated from Koytsoumpa et al.* [42]

Sector	**Process/ Product**
Chemical and Oil	Enhanced oil and gas recovery, enhanced coal bed methane recovery, stimulation/fracturing of oil and gas, urea production, polymer processing, chemicals and fuels
Food	Beverage carbonation, coffee decaffeination, wine production, food processing, food preservation, food packaging (modified or controlled atmosphere), dry ice production, horticulture (greenhouses), refrigeration
Mineralisation	Calcium and magnesium carbonate in cement, baking soda, bauxite residue treatment (red mud)
Power	Heat pumps, working medium in other CO_2 cycles
Energy crops	Algae cultivation
Pharmaceutical	Inerting, chemical synthesis, supercritical fluid extraction, product transportation
Others	Application in pulp and paper industry and in steel industry

1.3 Bioplastic and Polyhydroxyalkanoates (PHAs)

Among the wide varieties of bio-based products obtainable from biorefineries, as above mentioned, are the polyhydroxyalkanoates (PHAs), a class of bioplastics with microbial origin, which are completely biodegradable and are mainly produced starting from renewable sources, offering a wide range of physico-chemical properties that make them comparable to traditional plastics.[43]

Nowadays, the global plastics production amounts to 335 million tons, 18 % of which produced in Europe;[44] the high volume is the consequence of the growing demand for plastics, which in Europe is about 50 million tons per year.[44] The continuous demand for plastics on the market is due to the particular characteristics of this class of materials: low price (usually lower than 1 € kg^{-1}),[45,46] durability, lightness and ductility, which are all factors that make plastics versatile and utilizable in many commercial areas. On the other hand, "traditional" plastics which are not biodegradable lead to the accumulation of wastes that are liable to remain in the environment for hundreds/thousands of years. This problem could be partly solved by the recycling of these wastes, since plastics are completely recyclable. Currently only a small amount of these wastes is effectively recycled: in Europe only the 31.1 % of plastic wastes.[44] A possible alternative to traditional plastics are bioplastics, which include all the families of plastic materials that are bio-based or biodegradable, or both.[47] To face the growing demand for more sustainable alternative solutions to traditional plastics, the production capacity for bioplastics is increasing, although it still represents less than 1 % of the global plastics production.[48] The main limits that slow down bioplastics production are the high production costs and their physical properties that do not make them competitive on the market.

Therefore the possibility to produce bioplastics able to substitute traditional plastics in terms of thermo-mechanical behavior, involving low production costs, is now challenging: in this context, polyhydroxyalkanoates production from low-cost carbon sources is earning great interest.

<u>PHAs</u>

Polyhydroxyalkanoates (PHAs) are polyesters of several R-hydroxyalkanoic acids, fully bio-based and biodegradable, which are stored as carbon and energy reserves or reducing power storage materials by numerous microorganisms (Gram-negative and Gram-positive bacteria) in the presence of excess of carbon source, especially when another essential nutrient such as oxygen, nitrogen or phosphorus is limiting. When a limiting nutrient is provided to the cell, these energy storage compounds can be mobilized and degraded to be used for bacterial

growth.[43,49] PHAs are accumulated inside the cytoplasm under the form of granules that are typically 0.2 ± 0.7 μm in diameter and consist of 97.7 % PHA, 1.8 % protein and 0.5 % lipids.[50] Polyhydroxybutyrate (PHB) was the first PHA to be identified in 1926 by Maurice Lemoigne in cells of *Bacillus megaterium*;[51] today around 150 different PHA-building blocks, which have straight, branched, saturated, unsaturated and aromatic structures have been discovered [46] (general formula shown in Fig. 1.3) and over 90 genera of microbial species have been reported to accumulate these polymers [49].

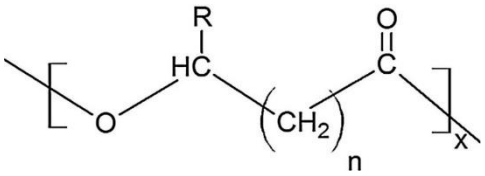

Figure 1.3 *Monomeric unity constituting PHAs (n= number of CH_2 in the linear chain; X= 100-30,000)*[47]

PHAs molecular weight can range from $1*10^4$ to $3*10^6$ Da [43] and, depending on the length of the monomers carbon skeleton, they can be classified into two groups: the short-chain-length (scl) PHAs, consisting of 3-5 carbon atoms and the medium-chain-length (mcl) PHAs, consisting of 6-14 carbon atoms.[49]

Examples of scl-PHAs are poly(3-hydroxybutyrate) P(3HB), poly(4-hydroxybutyrate) P(4HB), poly(3-hydroxyvalerate) P(3HV) or the copolymer P(3HB-co-3HV). Examples of mcl-PHAs include homopolymers such as poly(3-hydroxyhexanoate) P(3HHx) and poly(3-hydroxyoctanoate) P(3HO) and copolymers such as P(3HHx-co-3HO).

In general, PHAs monomeric composition depends on the strain, the substrate fed and the feeding mode, and their properties (i.e. the melting temperature, the glass transition temperature (Tg) and the crystallinity degree) are mainly determined by the composition, the microstructure (random or organized distribution of monomers) and the molecular weight distribution. In this way PHAs can range from brittle thermoplastics to gummy elastomers.[43]

Scl-PHAs are stiff, brittle with a high degree of crystallinity in the range of 60-80 %, while mcl-PHAs are flexible and elastic materials with low crystallinity (25 %), low tensile strength, high elongation to break, low melting temperatures and Tg below room temperature.[49,51]

Among PHAs family, the most interesting polymers and therefore the most studied are the P(3HB), the P(4HB), the P(3HB-co-3HV).

P(3HB) is highly crystalline, is water insoluble and relatively resistant to hydrolytic degradation. It has low O_2 permeability and good thermoplastic properties with poor

mechanical properties since it is highly brittle and stiff. While P(4HB) is a strong and malleable thermoplastic material with a tensile strength strictly comparable to that of polyethylene, it is also very elastic.[49]

The incorporation of secondary HA monomers other than 3HB (i.e. 3HV, 3HHx and 4HB) into the polymer chain is a common strategy. It greatly improves P(3HB) properties such as crystallinity, melting point, stiffness and toughness. The most well-known copolymer, P(3HB-co-3HV), has lower crystallinity, lower melting temperature, decreased stiffness, increased elongation to break, being more flexible than P(3HB).[49] In table 1.5 the main properties of P(3HB) and P(3HB-co-3HV) are shown and compared with polypropylene (PP), low and high density polyethylene (LDPE and HDPE, respectively).

Table 1.5 *Comparison of selected properties of P(3HB) and P(3HB-co-3HV) with polypropylene (PP), high density polyethylene (HDPE) and low density polyethylene (LDPE)* [45,46,48]

Property	**P(3HB)**	**P(3HB-co-3HV)***	**PP**	**HDPE**	**LDPE**
Mw (10^5 g mol^{-1})	1-8	3	2.2-7	-	-
ρ (kg dm^{-3})	1.25	1.2	0.905	0.941-0.965	0.91-0.925
Melting point (°C)	171-182	75-172	176	112-132	88-130
Crystallinity (%)	80	55-70	70	60-80	25-50
Glass transition temperature (°C)	5-10	-13-8	-10	-80	-36
O2-permeability (cm^3 m^{-2} kPa^{-1} day^{-1})	0.4	not determined	17	-	-
UV-resistance	good	good	bad	-	-
Resistance to solvents	bad	bad	good	-	-
Tensile strength (MPa)	40	25-38	38	17.9-33.1	15.2-78.6
Elongation to break (%)	3-8	8-1,200	400	12-700	150-600
Young´s modulus (GPa)	3.5-4	2.9 (3 % 3HV); 0.7 (25 % 3HV)	1-1.7	0.4-1	0.05-0.1

*for molar fraction of 3HV in the range of 4-95 %.

Beyond their physico-chemical properties, it has to be underlined that PHAs are completely recyclable, biodegradable and biocompatible.

In particular, PHAs can be degraded to H_2O and CO_2 under aerobic condition, while to H_2O, CH_4 and CO_2 under anaerobic condition, by microorganisms present in soil, sweet and salty water and in sewage. These microorganisms are able to produce extracellular PHAs depolymerases that hydrolyse the polymer into water-soluble oligomers and monomers, used then as carbon source by many organisms.[52] In particular, PHAs biodegradation can take about 1.5 months in anaerobic sewage, 1.5 years in soil, and 6.5 years in seawater.[53]

In animal tissues PHAs can be degraded by both enzymatic and non-enzymatic hydrolysis and their biocompatibility is due to the absence of any release of toxic substances, as well as by the

shape and the surface porosity of the material; for these reasons PHAs can be applied in medical field.[49,52]

PHAs applications

Thanks to their properties, PHAs have many application fields: packaging, medical area, biomedical engineering and agriculture.

One of the most useful applications of PHAs is the production of objects for food contact, which at the end of their life cycle can be collected together with food waste. Since 1996, the P(3HB-co-3HV) has received the European approval for food contact.[54] PHAs can also replace traditional plastics for the production of everyday items, such as shampoo bottles, cosmetic containers, transparent films for the production of compostable bags, coatings for paper objects, disposable products such as razors, plates, glasses, diapers, caps and lids.[49]

In the medical field PHAs are applied in the surgical, orthopaedic and cardiovascular areas for the production of repair devices and prosthesis. PHAs are also used to produce drugs and hormones delivery carriers.[55]

For what concern PHAs application in the environment, they can be used to produce mulching films for agricultural purpose or systems for the slow release of fertilizers, herbicides and insecticides.[49]

Moreover, due to their piezoelectric nature, it is possible to use PHAs for making pressure sensors for keyboards, stretch and acceleration measuring instruments, microphone and sound pressure measuring instruments.[49]

At a research level, PHAs can be used as biofuels in form of the hydroxyalkanoate methyl esters (3HAME), as fine chemical precursors under the form of pure enantiomeric monomers in (R)-configurations, as health food additives and therapeutic drugs in form of PHA monomers.[55]

PHAs production by pure microbial cultures

Many biotechnological industries dedicated a great effort in PHAs production from pure microbial cultures, generally performed in fed-batch mode: first, by adopting optimal growth conditions to promote the achievement of high cell density, then by exposing the culture to limiting growth conditions in order to produce PHAs.[43]

PHAs biosynthesis from pure cultures needs to be carried out in sterility and requires a close monitoring of the operating conditions (i.e. pH, temperature, media composition) to ensure an optimal growth for the specific strain, thus increasing the production costs. However, it remains

the most widespread method since it is characterized by the highest process yields (80-90 % PHAs on cell dry weight) [56] and the highest volumetric productivities [55].

PHAs production by mixed microbial cultures (MMCs)

Although pure cultures showed the highest volumetric productivities (up to 5 g PHB L^{-1} h^{-1}), the costs are too high to represent an economically viable option for polymer production.[43] One of the strategies adopted to reduce PHAs production costs is the use of mixed microbial cultures (MMCs), which mainly do not require sterility and strict control of growing conditions, resulting in both energy and economic savings during the fermentation process.[43,56]

Moreover, MMCs are more able than pure cultures to grow on complex substrates, such as low-cost agro-food industry waste, even to easily adapt to change in the substrate fed, resulting in a further possibility to reduce PHAs price [57] (nowadays it is around 4-6 € per kg) [58,59].

Mixed microbial cultures are microbial consortia operating in open biological systems, whose composition depends directly on the substrate and on the operating conditions imposed. It was observed that the activated sludge from the oxidation tank of wastewater treatment plants showed a significant amount of microorganisms able to accumulate PHAs, thus it is often used as inoculum to produce MMCs rich in PHAs producing bacteria.[43]

The most known process performed to enrich MMCs in PHAs accumulating bacteria is based on nature's principles of selection and competition ("evolutionary engineering"), achieved by applying selective pressure for a desired metabolism on a microbial consortium by adopting appropriate feeding and operating conditions.[60] Since PHAs are carbon storage compounds, naturally accumulated under dynamic conditions, cyclic feast-famine regime can be used as efficient selective pressure for a PHAs storing microbial cultures (Fig. 1.4). Such regime, called "aerobic dynamic feeding" (ADF), consists of constantly alternating availability (feast phase) and absence of substrate (famine phase), in aerobiosis.[60,61]

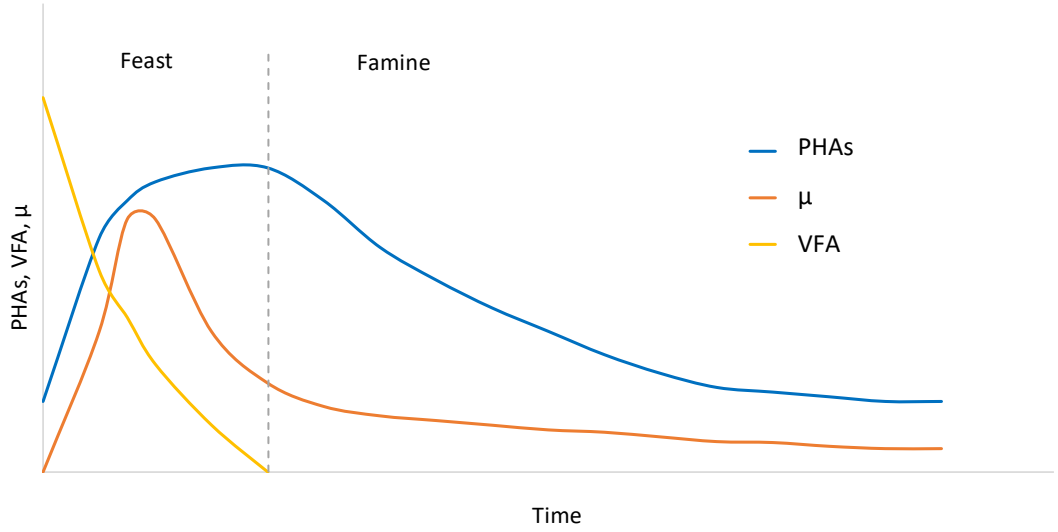

Figure 1.4 *Mechanisms for PHAs storage by MMCs under ADF condition. VFA (volatile fatty acids); μ (specific growth rate)* [43]

The transient availability of substrate induces a limitation in the internal growth of cells, indeed, after a period of absence of an external carbon source (famine phase), a reduction in the amount of intracellular molecules needed for growth (RNA and enzymes) is registered. After each famine phase, the need for physiological adaptation, which is the synthesis of the intracellular components required for growth, represents the main trigger mechanism of PHAs accumulation, since fewer enzymes are needed for PHAs storage. In this way PHAs accumulation can be performed at a much higher rate than cell growth, thus supplying the cells with a means of fast consumption of the external carbon source. After the external substrate is consumed, the internal PHAs storage is used as carbon and energy source for cell growth and maintenance.[43]

PHAs production by adopting MMCs is thus usually performed as a two-stage process,[43] where the first stage consists in the selection of a mixed microbial culture with high PHAs storing ability, while the second stage is represented by the massive polymer accumulation by the selected MMC, as it is shown in figure 1.5.

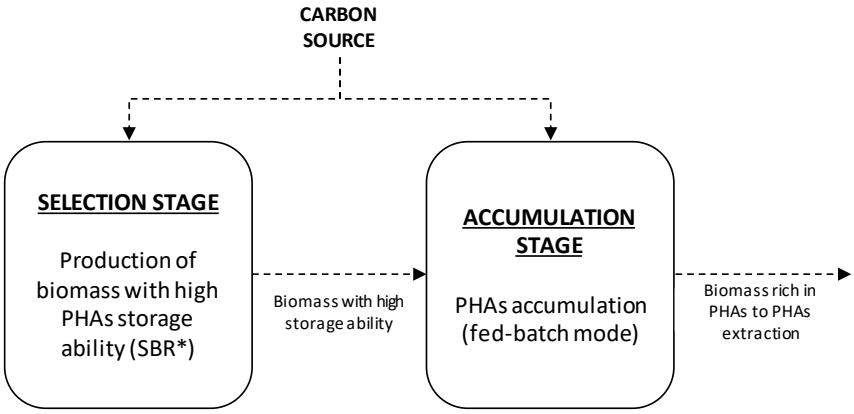

Figure 1.5 *Typical scheme of a two-stage process for PHAs production by MMCs. *SBR: sequencing batch reactor*

PHAs biosynthesis

As said before, many microorganisms show the capability of producing PHAs, which are accumulated inside the cell when the growing rate is lower than the substrate up-take rate, as a consequence of an external limitation (N, P or O_2) or of an internal limitation in anabolic enzyme levels or activity.[43]

Indeed, under growth-limiting conditions (i.e. N limitation), the acetyl-CoA produced from pyruvic acid after glycolysis, instead of being directed to the TCA (tricarboxylic acid) cycle, is addressed towards PHAs pathway. TCA cycle inhibition is caused by NADH and NADPH accumulation, which is the direct consequence of the decrease of proteins production due to the lack of an external nitrogen source.[43]

PHAs biosynthesis pathway involves three enzymes, which, in the particular case of P(3HB) synthesis, are: the 3-ketothiolase (PhaA), able to catalyse the condensation of two molecules of acetyl-CoA producing acetoacetyl-CoA, the acetoacetyl-CoA reductase (PhaB), that reduces acetoacetyl-CoA into (R)-3-hydroxybutyryl-CoA, and finally the PHA synthase (PhaC), which incorporates (R)-3-hydroxybutyryl-CoA into the polymer chain as 3HB.[43]

As well as pyruvic acid, that is the direct precursor of 3HB monomer, short-chain organic acids can be activated to the corresponding acyl-CoA to be used for different HA monomers production; these acids are usually consumed by mixed microbial cultures whose biosynthesis pathways have been assumed to be similar to those described for pure cultures by using the same substrates.

As it is shown in figure 1.6, other substrates (i.e. medium and long-chain fatty acids and alkanes) can be utilized to produce PHAs, following different metabolic pathways.[43]

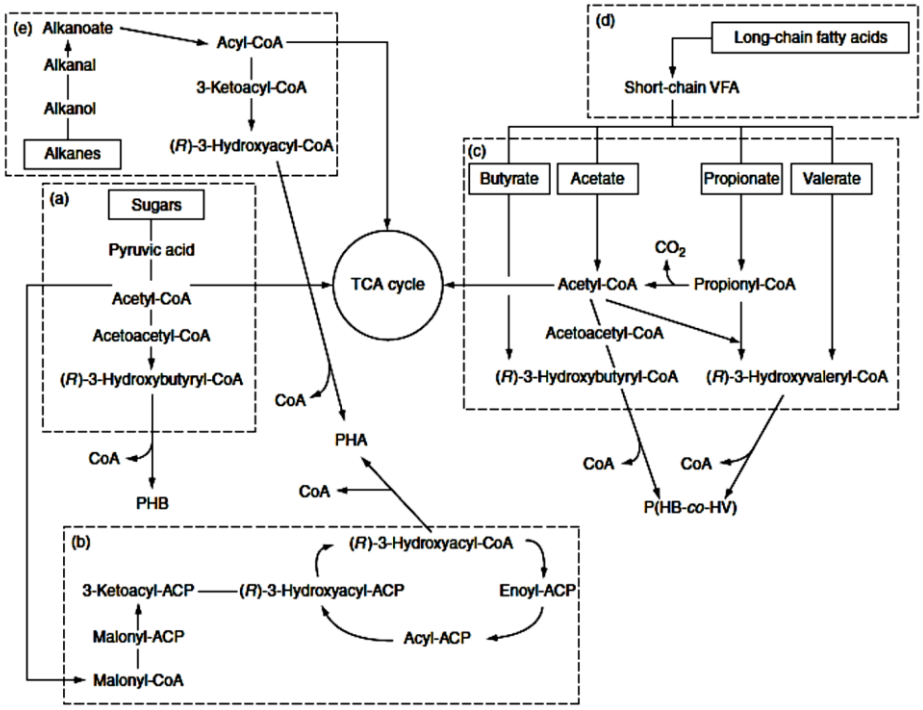

Figure 1.6 *Metabolic pathways involved in PHAs production from sugars, through glycolysis (a) or de novo fatty acid biosynthesis (b); from fatty acids, directly (c) or through fatty acid β-oxidation (d); and from alkanes, through alkane oxidation* [42]

PHAs production by MMCs from complex carbon streams

At an industrial scale, PHAs production is limited by the high production costs mainly associated to the substrate used [62] (30-40 % of the total production costs)[63] and to the downstream processes [50] (30-50 % of the overall production costs)[59,64]. The possibility of producing PHAs by MMCs, as said before, is more energy saving than by involving pure microbial strains, thus partly reducing the production costs; moreover MMCs are able to use complex carbon sources, such as agro-food industry by-products and food waste, thus overcoming the high cost derived from the use of pure standard substrates (i.e. glucose and starch). These complex organic streams need to be opportunely pre treated in order to produce suitable substrates for PHAs production by MMCs. Since organic acids have been reported to be the main precursors used by MMCs to synthetize PHAs,[43,53,65] acidogenic processes are usually adopted as complex carbon streams pre treatment before PHAs biosynthesis by MMCs.

Table 1.6 reports several examples of PHAs production by MMCs from fermented complex organic substrates.

Table 1.6 *Examples of PHAs production by MMCs by using fermented complex carbon streams as substrates*

Fermented substrate	OLR[a]	C/N[b]	HRT[c] (h)	SRT[d] (d)	Cycle lenght (h)	T (°C)	pH	Polymer composition (HB:HV) (%, w/w)	PHA_{max}[e] (%)	PHA yield	PHA productivity	Ref.
	PHAs accumulating bacteria selection reactor							*PHAs accumulation reactor*				
Cheese whey	1.9±04 (g COD L^{-1} d^{-1})	100/10 (C mol/ N mol)	24	4	12	23-25	8	81:19	65	0.67±0.13 (g COD_{PHA} g^{-1} COD_{FP}[f]$_{-cons.}$)	0.56 (g PHA L^{-1} h^{-1})	66
Cheese whey	8.5 (g COD L^{-1} d^{-1})	-	24	1	2	25	7.5	83:17-85:15	-	0.4 (g COD_{PHA} g^{-1} COD_{OA}[g]$_{-cons.}$)	-	67
Cheese whey	0.1 (C mol L^{-1} d^{-1})	100/4 (C mol/ N mol)	24	4	12	23-25	8.1-8.9	87:13	~32	0.96±0.07 (mmol C_{PHA} $mmol^{-1}$ $C_{FP-cons.}$)	-	68
Cheese whey	1.6 (g COD L^{-1} d^{-1})	100/5.8 (g COD/ g N)	24	4	12	30	-	94.9:5.1	51	0.75 (g COD_{PHA} g^{-1} $COD_{cons.}$)	-	69
Sugar cane molasses	2±0.6 (g COD L^{-1} d^{-1})	100/10 (C mol/ N mol)	24	4	12	23-25	8	48:52	56	0.85±0.19 (g COD_{PHA} g^{-1} $COD_{FP-cons.}$)	0.37 (g PHA L^{-1} h^{-1})	66
Sugar cane molasses	-	100/10 (C mol/ N mol)	32	10	8	30	<7.7	70:25:5(HHx)	37	0.47 (mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	-	61
Sugar cane molasses	3.3 (g COD L^{-1} d^{-1})	100/8 (C mol/ N mol)	24	10	12	23-25	8±0.05	74:26-83:17	75±5	0.66±0.03 (mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	-	70
Candy bar factory wastewater	0.054 (C mol L^{-1} d^{-1})	100/4 (g COD/ g N)	24	1	12	30	6.5-7.5	84:16	76	0.5 (g PHA g^{-1} $COD_{OA-cons.}$) 0.22 (g PHA g^{-1} $COD_{etOH-cons.}$)	12 (g PHA L^{-1} d^{-1})	71
Brewery wastewater	-	-	-	-	-	-	-	-	38	0.59 (mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	-	72
Olive oil mill effluent	8.5 (g COD L^{-1} d^{-1})	100/4 (g COD/ g N)	24	1	2	25	7.5	89:11	54	1 (g COD_{PHA} g^{-1} $COD_{OA-cons.}$)	-	73
Olive oil mill pomace	5.5 (g COD L^{-1} d^{-1})	-	24	6	-	20-25	-	-	39*	0.02* (g PHA g^{-1} $COD_{cons.}$)	0.042* (g PHA L^{-1} h^{-1})	74
Palm oil mill effluent	-	260 (g COD/ g N)	12	-	6	30	7±0.1	-	44.5±8.4*	-	0.127±0.09* (g PHA L^{-1} min^{-1})	75
Palm oil mill effluent	1.8 (g COD L^{-1} d^{-1})	100/20 (C mol/ N mol)	48	2	24	28-30	7	77:23	64	0.59 (g C_{PHA} g^{-1} $C_{OA-cons.}$)	-	76
Bio-oil from chicken beds pyrolysis	2 (g COD L^{-1} d^{-1})	100/1.7-100/5 (g COD/ g N)	24	5	12	20-23	7.2	73:27	16.83	0.58 (mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	-	77
FW	-	57 (g COD/ g NH_4)	12	2-12	6	20	-	77:23	60	-	-	78
OFMSW	6.4 (g COD L^{-1} d^{-1})	100/8	-	-	12	-	8	61:39	23.7±1	0.17 (g COD_{PHA} g^{-1} $COD_{cons.}$)	-	79
OFMSW Leachate[h]	3.9 (g COD L^{-1} d^{-1})	100/8 (g COD/ g N)	24	1	12	30	7±0.1	-	78.4	-	-	80
OFMSW Leachate[i]	5.24 (g COD L^{-1} d^{-1})	100/11 (g COD/ g N)	24	1	12	30	7±0.1	-	77.8	-	-	80
Paper mill wastewater	4.5 (g COD L^{-1} d^{-1})	100/10.7 (g COD/ g N)	48	2	24	30	7	86/14	76.8	0.8 (g COD_{PHA} g^{-1} $COD_{cons.}$)	2	81
Paper mill wastewater	-	-	-	7	-	30	7.3	39/61	68.8	0.66 (mmol C_{PHA} $mmol^{-1}$ $C_{OA-cons.}$)	-	82
Wood mill effluent	0.068 (C mol L^{-1} d^{-1})	26 (C mol/ N mol)	24	7	12	-	-	81/19	27	0.35 (mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	-	83

[a]OLR: organic loading rate;
[b]C/N: carbon to nitrogen ratio;
[c]HRT: hydraulic retention time;
[d]SRT: sludge retention time;
[e]PHA_{max}: expressed as amount of PHA on cell dry weight;
[f]FP: fermented products;
[g]OA: organic acids;
[h]accumulation test with a biomass selected with a substrate made of 90% synthetic VFA and 10% OFMSW leachate;
[i]accumulation test with a biomass selected with a substrate made of 75% synthetic VFA and 25% OFMSW leachate;
*values related to the selection reactor.

As it is shown in table 1.6, many studies demonstrate the possibility to achieve both high polymer content and conversion yield by using MMCs and fermented complex carbon streams to produce PHAs, thus determining a growing interest from biotechnology industries in substituting pure strains with mixed microbial consortia. In particular, considering cheese whey as substrate, Ahn et al.[84], by using *Escherichia coli* GCSC4401, obtained a PHAs content of 87 % on cell dry weight, 30 % higher than that reported by Duque et al.[66] by using a mixed culture able to convert about the 70 % of the fermented substrate into PHAs.

Concerning the use of molasses, Liu et al.[85], by using *Escherichia coli* HMS174, was able to reach a final polymer content on cell dry weight of 80 %, similar to what reported by Albuquerque et al.[70] by using fermented molasses as substrate for a MMC.

Regarding the use of more complex carbon sources, such as fermented food waste, both Omar et al.[86] and Hafuka et al.[87], by using two different strains of *Cupriavidus necator*, reported a PHAs content on cell dry weight of 86 % and 87 % respectively, not so higher with respect to what reported by Korkakaki et al.[80], by using the same substrate to feed a MMC.

Nowadays, the ability of mixed microbial consortia in producing PHAs from low-cost carbon streams has been confirmed by several investigations, on the other hand more energy and efforts have to be spent in order to increase the efficiency of the process (i.e. cell density and PHAs productivity). The integration of MMCs PHAs production into a biorefinery concept can be considered as a way to improve its economic and environmental performance.[88]

In this PhD thesis, PHAs production process from MMCs was integrated as a key step of four biorefinery approaches, aimed respectively to cheese whey, OFMSW, *Arundo donax* and CO_2 valorization.

For all of the biorefineries, an acidogenic process was adopted as the first step of the carbon stream treatment, focused on organic acids biosynthesis (i.e acetic, propionic, lactic, butyric and valeric acids), in order to convert the raw carbon stream into an adequate substrate for the successive PHAs production step from MMCs.

PHAs extraction from MMCs

In the end of this work, studies aimed to the investigation of alternative PHAs extraction methods from MMCs have been performed.

In PHAs production the downstream process plays a key role in the profitability of the fermentation system: nowadays it is known to count for about the 30-50 % of the total production costs,[59,64] due to extensive use of non-recyclable (and sometimes highly toxic) chemicals/materials and due to the high energy consumption [89].

Downstream strategies consist mainly in PHAs granules extraction from the bacterial cells, in some cases preceded by pre treatments in order to get a better recovery yield and/or followed by a purification step to get a higher purity of the polymer.

The ideal method should lead to a high purity and recovery level at a low production cost.[90]

The extraction procedures currently adopted can be divided into two classes: PHAs solubilisation/recovery with organic solvents and the dissolution of Non-PHA Cell Mass (NPCM) with chemicals (i.e. acids, alkalis and surfactants) or enzymes.[59,91]

However, both the approaches present several disadvantages:

- Chlorinated organic solvents, such as chloroform and dichloromethane, are the best performing, but since they create hazards for the operators and the environment, they still do not represent a good choice at an industrial level. Other more sustainable solvents have been tested (i.e. dimethyl carbonate - DMC, propylene carbonate, ethyl acetate, methyl isobutyl ketone), reporting good results in terms of purity and recovery yields, but negative impact on the molecular weight of the final polymer, due to the high temperature required. Moreover, other "green" alternatives, such as ionic liquids and supercritical fluids, are still too costly to represent a valid substitute.[59]
- Concerning NPCM dissolution with chemicals, at the beginning nonselective dissolution systems were adopted, mainly represented by alkalis (i.e. NaOH and NaClO) and acids digestion, which if too concentrated can determine a degradation of both NPCM and PHAs, thus reducing the recovery yield and lowering PHAs molecular weight. These methods were then replaced by selective dissolution chemicals, including anionic (i.e. sodium dodecyl sulfate - SDS), cationic (i.e. hexadecyltrimethylammonium bromide - CTAB), non-ionic surfactants (i.e. Tween 20 and Triton X-100) and proteolytic enzymes. For what concern the use of surfactants, all of them can be directly applied to the wet microbial culture, thus avoiding the expensive dewatering step, and they do not affect the properties of the final product. On the other hand, to work

properly, a huge amount of surfactant is needed, determining high wastewater production to be necessarily recovered in order to contain the costs. Another drawback of this procedure is the low purity of the extracted PHAs, that often require a final purification step.[59,91]

The downstream strategies above mentioned are the ones mainly performed to recover PHAs from pure microbial strains; in the last few years such processes have started to be adapted for PHAs extraction from mixed microbial consortia, but still few information are available on their efficiency. With respect to pure cultures (in which genetic modification and/or cell constrains, due to high polymer amount, increase cellular fragility), MMCs are more resistant to cell hydrolysis, thus determining the need of greater efforts in PHAs recovery.[89]
In table 1.7, examples of downstream procedures adopted to extract PHAs from MMCs have been reported.

Table 1.7 *Examples of downstream processes to recover PHAs from MMCs*

Biomass	Pre treatment	Chemicals	Operating condition	Purification	Recovery yield (%)	Purity (%)	Average Mw (MDa)	Polydispersity Index	Ref.
Freeze-dried MMC fed with mix of organic acids	-	CH_2Cl_2	50 °C, 4 h	-	52±1	94	1.4	2	[89]
Freeze-dried MMC fed with mix of organic acids	-	DMC	90 °C, 1h	-	49±2	98	1.3	1.9	[89]
Dry MMC fed with acetate	Refluxing with acetone (300 mL, 3h)	CH_2Cl_2:H_2O (8:1)	Refluxing, 30 min	Precipitation with hot H_2O	30	-	2.2	1.3	[92]
Freeze-dried MMC fed with mix of organic acids	NaClO (5 % Cl_2) (15 min)	DMC	90 °C, 1h	-	62±4	98	0.8	2.4	[89]
Freeze-dried MMC fed with mix of organic acids	NaClO (5 % Cl_2) (1 h, 100 °C)	DMC	90 °C, 1h	-	82±3	93	0.2	2.5	[89]
Freeze-dried MMC fed with palm oil mill effluent	-	$CHCl_3$: NaClO (6 % Cl_2) (1:1)	37 °C, 3 h, 300 rpm	Precipitation with MeOH (9:1) and oil saponification with hexane	>90	>90	1.8-2.2	2.5-3	[90]
Concentrated MMC fed with acetate	Preheating (30 °C)	NaOH (0.2 M) + SDS (0.2 %)	30 °C, 1 h, 100 rpm	Washing with H_2O	91.0±4.9	99.1±0.5	0.48	-	[93]
MMC fed with mix of organic acids	-	NaClO (5 % Cl_2): slurry (1:6)	room T, 24 h	-	100±5	98±5	0.34-0.54	4-10	[94]
Freeze-dried MMC fed with mix of organic acids	-	SDS (0.12 M)	90 °C, 3 h	Washing with H_2O and EtOH	67.1±4.0	56.1±6.6	-	-	[89]

As can be observed from table 1.7, several methods were applied to recover PHAs from MMCs, reporting differences in terms of yield, purity and molecular weight of the extracted polymer.

It can be seen that by using a sole organic solvent, both high purity and high molecular weight in the final polymer can be obtained, while the recovery yield is still low with MMCs.

The introduction of a pre treatment step with strong oxidants (NaClO, NaOH) before solvent extraction or the sole use of oxidant chemicals can help in increasing the recovery yield, on the other hand it determines a great reduction in the molecular weight of the final polymer, thus affecting its successive application.

Only Salmiati et al.[90] reported good results in terms of yield, purity and molecular weight by using a mixture of NaClO and chloroform to extract PHAs from a MMC cultivated on palm oil mill effluent.

Therefore, more efforts have to be spent in order to find an optimized method for PHAs recovery from MMCs.

After PHAs extraction, the possibility to valorize the exhausted microbial biomass for the production of valuable bioproducts or biofuel has to be taken into account. For this reason, the NPCM collected from the downstream process has to be mostly free of hazardous residues.

Three different destinies can be proposed for it: biogas production through anaerobic digestion, the production of C-N-rich sources obtained by chemical or enzymatic hydrolysis to be then used for microbial cultivations, and its application in agriculture as "green fertilizer".[91]

Considering all of these aspects, in this PhD thesis, a new PHAs recovery procedure from MMCs has been developed by performing the selective dissolution of NPCM with non-ionic surfactants as a pre treatment of PHAs extraction achieved by using non chlorinated and sustainable solvents, i.e. dimethyl carbonate - DMC.

2. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture

Colombo, B., Pepè Sciarria, T., Reis, M., Scaglia, B., Adani, F. (2016). Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. *Bioresource Technology*, vol. 218, p. 692-699.

Abstract

In this work two fermented cheese whey (FCW), i.e. FCW1 composed by lactic, acetic and butyric acids in the proportion of 58/16/26 (%, $COD_{Organic\ Acid}$) and FCW2 composed by acetic, propionic, butyric, lactic and valeric acids in the proportion of 58/19/13/6/4 (%, COD_{OA}) were used to produce polyhydroxyalkanoates (PHAs) by using a pre-selected mixed microbial culture (MMC). PHA accumulation gave for FCW1 a PHA yield (Y_{tot}) of 0.24 ± 0.02 mg COD_{PHA} mg $COD_{Soluble\ Substrate(SS)}^{-1}$ and a total PHA production, referred to the substrate used, of 60 g PHA $kg_{cheese\ whey}$ Total Solids $(TS)^{-1}$. For FCW2 the results were: PHA yield (Y_{tot}) of 0.42 ± 0.03 mg COD_{PHA} mg COD_{SS}^{-1} and PHA from substrate of 70 g PHA $kg_{cheese\ whey}$ TS^{-1}. Qualitatively, PHA from FCW1 was composed exclusively by polyhydroxybutyrate (PHB) contrarily to those obtained from FCW2 that were composed for 40 % of hydroxyvalerate (HV) and for 60 % of HB.

2.1 Introduction

In 2014 the global production of plastics amounted to 311 million Mg;[95] in particular, in the same year, in Europe 59 million Mg of plastics were produced,[95] the high volume being the consequence of the growing demand for plastics which in Europe is equal to 47.8 million Mg annually.[95]

The continuous demand for plastics on the market is due to the particular characteristics of this class of materials: low price (usually lower than 1 € kg^{-1}),[45] durability, lightness and ductility, which are all factors that make plastics versatile and utilizable in many commercial sectors.[96] On the other hand, ‘‘traditional” plastics which are not biodegradable, lead to the accumulation of wastes that are liable to remain in the environment for hundreds of years. This problem could be partially solved by the recycling of these wastes, since plastics are completely recyclable. Nowadays only a small amount of these wastes is effectively recycled: in 2014, in Europe, only 29.7 % of plastic wastes were recycled.[95]

A possible alternative to traditional plastics are bioplastics, which include all the families of plastic materials that are bio-based or biodegradable, or both.[97]

To face the growing demand for alternative solutions to traditional plastics, the production capacity for bioplastics is increasing, although in 2014 the global production of these products was less than 1 % of global plastics production. The previsions for 2019 indicate that the annual production of bioplastics should reach almost 7.8 million Mg.[98]

The main limits that slow down bioplastics production are the high production costs and their physical properties that do not make them competitive on the market.

Among the renewable raw materials used for bioplastics production are starch, cellulose, lignin, proteins and many other carbon sources.[54] These materials can originate from both food crops, such as corn and sugar cane, and from municipal and agro-industrial wastes. Many of the bio-based plastics originate from these substrates and, among them, polyhydroxyalkanoates (PHAs) are of particular interest, since they present a wide range of thermo-mechanical properties that make them potential substitutes for conventional polyolefins. Moreover, these biopolymers are fully sustainable because they can be produced from renewable sources and they are completely biodegradable.[43]

PHAs are bio-polyesters produced by different bacterial genera from various carbon sources, and they are accumulated in bacterial cells under the form of granules as carbon and energy storage.

PHAs can be produced by both pure and mixed microbial cultures (MMCs): pure cultures are associated with the best process yields and PHA productivities, because they are chosen for their high storage capacity and high cell density.[43] On the other hand, mixed cultures, before PHAs production, have to be enriched with PHAs-storing microorganisms. Mixed cultures have the advantage that they do not need sterile conditions and they are better able than pure cultures to adapt to complex substrates such as agro-industrial wastes.[43]

Various authors have used diverse substrates to produce PHAs from MMC, such as molasses,[99] palm oil mill effluent,[100] tomato cannery wastewater,[101] olive oil mill effluents,[73] food waste[78] and cheese whey[66,67,102].

Cheese whey is the main waste product of the dairy industry; every year 120 million Mg of cheese whey are produced in the world, 66 % of which are generated in the EU and 25 % in North America.[10] Cheese whey is a green-yellowish liquid resulting from the precipitation and removal of milk casein in the cheese making process. It has a high carbon load (COD= 50-102 g L^{-1}) and it is composed of lactose (39-60 g L^{-1}; about 90 % of the organic load), fat and

proteins (0.99-10.58 g L^{-1} and 27-60 g L^{-1}, respectively), and mineral salts (4.6-8 g L^{-1}), mainly represented by NaCl, KCl and calcium phosphates.[11]

To minimize the amount of cheese whey to be annually disposed of, different strategies have been developed and adopted aimed at the production of energy or goods with high benefits.[11] These actions transform cheese whey into products of interest and at the same time generate low carbon load effluents to reduce the environmental impact.[11]

In particular, the presence of the disaccharide lactose in cheese whey makes it an interesting carbon source which can be used either directly as the substrate for polyhydroxyalkanoates production[102] or after fermentation of the whey[66,67].

In the Lombardy Region (northern Italy) about 3.3 million Mg of cheese whey (data of 2013) are produced yearly, that is about 36 % of the total cheese whey produced in Italy and 2.75 % of the global production.[103] This huge amount of cheese whey is only partially used (about 50 %) directly as animal feed or to produce powder for feed and food,[13] and Ricotta cheese, the remaining part not being traceable or cleaned.[14] These numbers suggested that cheese whey may be a suitable substrate to produce PHAs,[104] addressing, also, the potential of economic investment in this research (e.g. Integrated Research on Industrial Biotechnologies and Bioeconomy – Cariplo Foundation, 2015).

Since few authors have reported on the use of cheese whey for PHAs production by mixed microbial cultures, the aim of this study was a first attempt to investigate the use of fermented cheese whey to produce PHAs by using a selected mixed microbial culture. PHA composition and yields are reported and discussed in view of possible future applications.

2.2 Materials and methods

Inoculum

The enrichment of PHAs-producing bacteria was performed by using an inoculum consisting of an activated sludge (8 g Total Suspended Solids - TSS L^{-1}) collected from the secondary sedimentation tank of a wastewater treatment plant ($5.2*10^5$ equivalent inhabitants) located at Peschiera Borromeo (Milan, Italy).

Cheese whey fermentation procedure

Substrates for the selection of PHAs-accumulating bacteria and for the PHA accumulation tests were obtained by carrying out two different cheese whey fermentations. The first fermentation was set up with fresh cheese whey (cheese whey 1 - FCW1) in order to achieve a high percentage of lactic acid in the final fermented medium thanks to the presence in the substrate of autochthonous lactic bacteria. The second fermentation test was performed by using

sterilized cheese whey (cheese whey 2 - FCW2) (120 °C for 20 min), subsequently inoculated with sporogenous acidogenic bacteria coming from the liquid fraction of digestate (50 g TSS L^{-1}; 35 g Volatile Suspended Solids - VSS - L^{-1}) collected from an anaerobic digestion plant, then heat-shocked at 100 °C for 1 h (1:5 ratio, inoculum: cheese whey). The two 96-h fermentation tests were carried out in two glass flasks with a working volume of 500 mL, mechanically stirred at 150 rpm, with a pH set at 5.5 corrected automatically by adding 3 mol L^{-1} KOH in a lab-incubator kept at 37 °C. The two flasks were fluxed with N_2 and kept closed to maintain anaerobic conditions inside.

Before their use as substrates for PHA production, the two fermented cheese wheys were heated in a closed bottle at 100 °C for 30 min, then cooled and centrifuged at 8,385 g for 10 min, keeping the supernatant to be used as the substrate.

PHAs-producing bacteria enrichment

The enrichment in PHAs-producing bacteria was performed in a Sequencing Batch Reactor (SBR) with a working volume of 1 L, applying an aerobic dynamic feeding (ADF) strategy.[66] In brief the SBR cycle length was of 12 h, consisting of four discrete phases: (i) influent filling (4 min), (ii) aeration (675 min), (iii) settling (40 min), and (iv) withdrawal of the exhausted effluent (5 min), with 1 day of Hydraulic Retention Time (HRT) and 5 days of Sludge Retention Time (SRT), keeping the temperature at 25 °C and the pH at 8.8, this latter controlled automatically by adding 1 mol L^{-1} HCl. Aeration and agitation were provided by supplied air at 6 L min^{-1} and stirring set at 110 rpm. Pumping, aeration and stirring were automatically controlled. The selection trend was monitored by determining the duration of both feast and famine phases achievable by using the dissolved oxygen (DO) concentration in the selection media[66] measured by an optical probe (FDO 925, WTW, Germany). Reis et al.[43] reported that the ability to produce PHA demonstrated by microbial cultures operated under feast and famine (FF) conditions comes from the internal growth limitation caused by transient substrate availability. After a period of time in absence of external carbon source, a decrease of the amount of intracellular components needed for cell growth occurs. Hence, when carbon source becomes available again, the amount of growth enzymes may not be enough to ensure a maximum growth rate. On the other hand, fewer enzymes are required for PHA accumulation and, therefore, PHA storage can occur at a faster rate, providing the cells with a means of rapidly consuming the available external substrate. After that external carbon is depleted, the internal PHA storage serve as energy source for cell growth and maintenance.

In particular, the feast (h) to famine (h) ratio (F/F ratio) was calculated as the ratio between the length in hours of the two phases. As reported by Valentino et al.[45] for a correct selection of PHAs-storing bacteria the F/F ratio had to be equal or less than 0.33.

Three different selection tests were performed; the first test was performed by using 500 mL of activated sludge used as inoculum fed, for each cycle, with 500 mL of synthetic medium,[66] prepared with CH_3COONa (1.395 g L^{-1}), NH_4Cl (0.214 g L^{-1}), KH_2PO_4 (0.054 g L^{-1}) and Allylthiourea (20 mg L^{-1}), this latter added to inhibit nitrification. Organic Loading Rate (OLR) was equal to 37 ± 2 mmole C L^{-1} d^{-1} and the C/N/P ratio was of 100/10/0.5 mmole C/mmole N/mmole P. The duration of this process was of 98 cycles. In particular, the first 8 cycles considered an OLR of 40 mmole C L^{-1} d^{-1} that was then reduced to an OLR of 34.2 mmole C L^{-1} d^{-1} to maintain the feast duration within 25-30 % of the total cycle duration[45]. The second and third trials were performed in order to test the selected PHAs-accumulating bacteria by using a fermented agricultural waste as carbon source. A new activated sludge (500 mL) was submitted to the selection procedure by using fermented cheese whey 1 (FCW1) as the substrate and adopting the operating conditions defined during the first test. In this case, due to the chemical composition of the substrate used, the C/N/P mmole ratio was of 100/12.3/3 and the OLR adopted was equal to 39.7 ± 2.4 mmole C L^{-1} d^{-1}. The FCW1 C/N/P mmole ratio was bigger than those calculated for the first test; however, the final concentration of N and P in the reactor (respectively of 34.2 mg N L^{-1} and of 18.5 mg P L^{-1}) were lower than the values considered to inhibit PHA storage (100 mg N L^{-1} and 50 mg P L^{-1})[105]. Because FCW1 contained larger amounts of N and P (respectively 68.36 mg N L^{-1} and 36.98 mg P L^{-1}) in comparison with those of the first feeding solution (47.9 mg N L^{-1} and 5.3 mg P L^{-1}) these elements were not added to the growth media. The culture was kept in these conditions for 40 cycles. The third test was performed to keep selected the mixed culture resulting from the second test, using the FCW2 as substrate. In the third test, depending on the chemical composition of the substrate utilized, the C/N/P mmole ratio and OLR were of 100/19.7/3.9 and of 38 ± 2 mmole C L^{-1} d^{-1}, respectively. In this case, also, the minerals needed for cell growth were already present in the FCW2, and the final nitrogen and phosphorus concentration in the reactor (N= 52.4 mg L^{-1} and P= 23 mg L^{-1}) were greater than those of the first test but lower than the values considered to limit PHA storage[105]. The culture was kept in these conditions for 31 cycles.

PHA accumulation procedure

The ability of the MMC to accumulate PHA was assessed by fed-batch assays carried out in a 500 mL working volume glass reactor, with continuous aeration and stirring. These assays

consisted in feeding the substrate to 200 mL of enriched culture (at least 3 SRTs from the beginning of the selection)[106] adopting a pulse-wise feeding method. The assays were monitored continuously by measuring the concentration of the dissolved oxygen in the accumulation media.[66] In particular, substrate was fed to the reactor when DO showed a strong increase.[66] Total C dosed was calculated taking into account that the ratio of the carbon to the microorganism had to be the same as that inside the selection reactor. The assays were stopped when no DO variation followed the substrate feeding. For the accumulation tests, the operating conditions adopted were those adopted in the selection reactor, i.e. temperature of 25 °C, aeration of 6 L min^{-1} and stirring at 110 rpm. The biomass selected with sodium acetate was submitted to accumulation tests using the same substrate as carbon source. However, the biomasses selected by using the FCW1 and FCW2, were submitted to accumulation tests by using the same substrates used for the selection tests. For each substrate, the accumulation trial was performed in duplicate.

Analytical procedures

Both substrates fed during the selection and accumulation processes were characterized in terms of pH, Total Solids (TS), Volatile Solids (VS), soluble Chemical Oxygen Demand, organic acids (OA) content (acetate, butyrate, lactate, propionate and valerate), nitrogen (N), ammonium ($N\text{-}NH_4$) and phosphorus (P) content. During the selection trials, samples were taken during the cycle once a SRT; every sample was characterized in terms of pH, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), soluble COD, organic acids content, $N\text{-}NH_4$ content and PHA content. During accumulation trials, samples were taken continuously in order to measure TSS, VSS, soluble COD, organic acids content and PHA content.

Biomass concentration was calculated as VSS according to the standard methods[66]. TSS and VSS were determined as reported by Valentino et al.[67]; phosphorus was determined by using international methods[107]. Organic acids concentrations measured on filtered samples (filter diameter of 0.45 μm) were determined by high performance liquid chromatography (HPLC) using a chromatograph equipped with an UV detector and Aminex HPX 87H column (column temperature 20 °C, 0.0025 M H_2SO_4 eluent, flow rate 0.6 mL min^{-1}). The organic acids concentrations were calculated through a standard calibration curve (20-1000 mg L^{-1} of each organic acid). The COD and the $N\text{-}NH_4$ content (filtered at 0.45 μm) were determined using cuvette test kits (Macherey-Nagel, Germany). PHAs were determined by GC MS using a method adapted from Serafim et al.[57]. Lyophilized biomass was incubated for methanolysis in

a 20 % v/v H_2SO_4 in MeOH solution (1 mL) and extracted with chloroform (1 mL). The mixture was digested at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was extracted and injected (1 mL) into a gas chromatograph equipped with a detector (7980, Agilent Technologies, USA) and a ZB-Wax column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness, Zebron, Phenomenex, USA), using helium as carrier gas at 1.0 mL min^{-1}. Samples were analysed under a temperature regime starting at 40 °C, increasing to 100 °C at a rate of 20 °C min^{-1}, to 175 °C at a rate of 38 °C min^{-1} and reaching a final temperature of 220 °C at a rate of 20 °C min^{-1} for ensuring cleaning of the column after each injection. Injector and detector temperatures were at 280 °C and 230 °C, respectively. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through the use of two calibration curves, one for HB and other for HV, using standards (0.1-8 g L^{-1}) of a commercial P (HB-HV) (88 %/12 %) (Sigma Aldrich, Germany), and corrected using heptadecane as internal standard (concentration of approximately 1 g L^{-1}) (Sigma Aldrich, Germany).

Results expression

Cheese whey fermentation test

Fermentation yield ($Y_{\text{Organic Acids (OA)/ Soluble Substrate (SS)}}$, mg COD_{OA} mg COD_{SS}^{-1}) was calculated as the ratio between the amount of organic acids produced (in terms of COD - COD_{OA}) and the amount of substrate depleted (in terms of soluble COD - COD_{SS}); fermentation yield was also calculated in terms of C mole basis, and expressed as mmole Organic Acids (OA-C) mmole Soluble Substrates $(SS\text{-}C)^{-1}$.

MMC selection and PHA accumulation tests

The PHA content in cells was reported as the percentage of VSS on a mass basis [PHA= (g PHA kg VSS^{-1})], considering VSS to be constituted by both active biomass (X) and PHA.[66] PHA were converted into COD according to the following oxidation stoichiometry: 1.67 mg COD mg HB $monomer^{-1}$ and 1.92 mg COD mg HV $monomer^{-1}$.[45]

Acetate, butyrate and lactate were considered as 3-hydroxybutyrate (HB) precursors, valerate and propionate as 3-hydroxyvalerate (HV) precursors.[66]

X was calculated on a C mole basis taking into consideration that 1 g of active biomass contains 44.2 mmole C;[66] X was also calculated on a COD basis considering that 1 g of active biomass contains 1.42 g of COD.[45]

Active biomass growth was calculated from the $N\text{-}NH_4$ depletion, as reported by Valentino et al.[45]. Specific organic acids consumption rate ($-q_{\text{OrganicAcids (OA)}}$, mmole OA-C mmole $X\text{-}C^{-1}$ h^{-1}

or mg COD_{OA} mg COD_X^{-1} h^{-1}) was determined as the ratio between the amount of organic acids consumed (mmole OA-C or mg COD_{OA}), and the time (h) needed to deplete them per unit of active biomass (mmole X-C or mg COD_X). Specific PHA storage rate (q_{PHA}, mmole PHA-C mmole $X\text{-}C^{-1}$ h^{-1} or mg COD_{PHA} mg COD_X^{-1} h^{-1}) was determined as the ratio between the amount of PHA stored (mmole PHA-C or mg COD_{PHA}) and the time (h) needed to deplete the organic acids per unit of active biomass (mmole X-C or mg COD_X). Specific growth rate ($q_{Active\ biomass\ (X)}$, mmole X-C mmole $X\text{-}C^{-1}$ h^{-1} or mg COD_X mg COD_X^{-1} h^{-1}) was determined as the ratio between the amount of active biomass growth (mmole X-C or mg COD_X) and the time (h) needed to deplete the organic acids per unit of active biomass (mmole X-C or mg COD_X), as reported by Valentino et al.[45].

For the SBR the storage yield ($Y_{PHA/Organic\ Acids\ (OA)}$, mmole PHA-C mmole $OA\text{-}C^{-1}$) was calculated as the ratio between the amount of PHA accumulated and the amount of substrate (organic acids) depleted and, also, on a COD basis (mg COD_{PHA} mg COD_{OA}^{-1}). Again the growth yield ($Y_{Active\ biomass\ (X)/Organic\ Acids\ (OA)}$, mmole X-C mmole $OA\text{-}C^{-1}$) was calculated as the ratio between the new biomass produced and the amount of organic acids depleted, calculated also on a COD basis (mg COD_X mg COD_{OA}^{-1}), as reported by Valentino et al.[45]. In the accumulation batches, the specific rates and yields were calculated as described before, for each pulse. In order to compare different accumulation tests, the average values for the first three pulses and for each parameter were considered.

Moreover, the total process yield ($Y_{PHA/Soluble\ Substrate\ (SS)}$) was calculated as a product of the fermentation yield ($Y_{OA/SS}$) and the storage yield ($Y_{PHA/OA}$), expressed on a C mole basis as mmole PHA-C mmole $SS\text{-}C^{-1}$, or expressed on a COD basis as mg COD_{PHA} mg COD_{SS}^{-1}.

2.3 Results and Discussion

Cheese whey fermentation

In the first fermentation test the conversion yield from the soluble substrate (FCW1) carbon to organic acids carbon was equal to 0.4 mmole OA-C mmole $SS\text{-}C^{-1}$; in terms of composition, the fermented FCW1 contained lactic acid, acetic acid and butyric acid in the proportion of 58/16/26 (%, COD_{OA} basis) (Table 2.1).

In the second fermentation test a conversion yield of 0.6 mmole OA-C mmole $SS\text{-}C^{-1}$ was obtained, and the fermented slurry (FCW2) was composed of acetic acid, propionic acid, butyric acid, lactic acid and valeric acid, in the proportion of 58/19/13/6/4 (% COD_{OA}).

Table 2.1 *Chemical composition of the substrates used in this study*

Substrate	**pH**	**TS** ($g\ L^{-1}$)	**VS** ($g\ L^{-1}$)	**Soluble COD** ($g\ L^{-1}$)	**N tot** ($mg\ L^{-1}$)	**P tot** ($mg\ L^{-1}$)	**C/N/P** (C/N/P mmole)	**OLR** (mmole C $L^{-1}\ d^{-1}$)	**Organic acids composition** (%, COD_{OA} basis)
Acetate	7.2	-	-	1.3±0.1	47.9±6.2	5.3±0.5	100/10/0.5	37±2	Acetate 100
FCW1	5.2	7±1	3.4±0.3	8.3±0.7	376±30	203±21	100/12.3/3	39.7±2.4	Lactate/Acetate/ Butyrate 58/16/26
FCW2	6	7±1	3.5±0.3	5.6±0.4	407±33	178±18	100/19.7/3.9	38±2	Lactate/Acetate/ Propionate/Butyrate/ Valerate 6/58/19/13/4

Mixed microbial culture enrichment and PHA production

Acetate as substrate

The first selection process showed a feast/famine ratio (F/F ratio) trend in the range of 0.2-0.3, with this value being taken to indicate a correct selection of PHAs-accumulating bacteria[45]. As reported in table 2.2 (Selection Process), the maximum PHA content at the end of the feast phase was of 420 g kg VSS^{-1}, and the polymer accumulated was represented entirely by PHB.

The specific organic acids consumption rate, the specific PHA storing rate and the specific growth rate were of 0.5 mmole OA-C mmole X-C^{-1} h^{-1}, 0.2 mmole PHA-C mmole X-C^{-1} h^{-1} and 0.14 mmole X-C mmole X-C^{-1} h^{-1}, respectively. Moreover, the storage yield was equal to 0.4 mmole PHA-C mmole OA-C^{-1} and the growth yield equalled to 0.25 mmole X-C mmole OA-C^{-1}.

Once the culture was enriched with PHAs-storing bacteria, an accumulation assay was performed in order to evaluate the maximum PHA storing capacity of the selected culture. The test was carried out in a 500 mL working volume glass-reactor, inoculated with 200 mL of the selected culture purged from the SBR. During the test, culture was fed in pulse-wise mode adding only the sodium acetate solution without mineral solution. This was done because it had been reported that N limitation determined a greater conversion of carbon in PHA because of cell growth limitation.[57]

As reported in table 2.2 (Accumulation Process), accumulation in PHA at the end of the test (Figure 2.1a) was equal to 824 g kg VSS^{-1}, and it was composed only by PHB; the storage yield was of 0.68 mmole PHA-C mmole OA-C^{-1} and the PHA productivity was equal to 0.9 g PHA $L^{-1}\ d^{-1}$.

Table 2.2 *Parameters characterizing the selection process and the accumulation assay with sodium acetate*

Selection Process									
Substrate	**F/F**[a] (h h^{-1})	**PHA_{max}**[b] (g PHA kg VSS^{-1})	**Polymer composition (ΔHB:ΔHV)** (%, W/W)	**$-q_{OA}$**[c] (mmole OA-C mmole X-C^{-1} h^{-1})	**q_{PHA}**[d] (mmole PHA-C mmole X-C^{-1} h^{-1})	**q_X**[e] (mmole X-C mmole X-C^{-1} h^{-1})	**$Y_{PHA/OA}$**[f] (mmole PHA-C mmole OA-C^{-1})	**$Y_{X/OA}$**[g] (mmole X-C mmole OA-C^{-1})	**X_{max}**[h] (g VSS L^{-1})
Acetate	0.2±0.0	420±30	100:0	0.5±0.0	0.2±0.0	0.14±0.01	0.4±0.0	0.25±0.0	0.6±0.0

Accumulation Process							
Substrate	**Test length** (h)	**$PHA_{end\ test}$**[i] (g PHA kg VSS^{-1})	**Polymer composition (ΔHB:ΔHV)** (%, W/W)	**$-q_{OA}$** (mmole OA-C mmole X-C^{-1} h^{-1})	**q_{PHA}** (mmole PHA-C mmole X-C^{-1} h^{-1})	**$Y_{PHA/OA}$** (mmole PHA-C mmole OA-C^{-1})	**PHA Productivity** (g PHA L^{-1} d^{-1})
Acetate	7.2±0.1	824±58	100:0	0.44±0.03	0.29±0.01	0.68±0.04	0.9±0.1

[a]F/F (feast to famine ratio);
[b]PHA_{max} (PHA at the end of the feast phase);
[c]$-q_{OA}$ (specific organic acids uptake rate);
[d]q_{PHA} (specific PHA accumulation rate);
[e]q_X (specific growing rate);
[f]$Y_{PHA/OA}$ (storage yield);
[g]$Y_{X/OA}$ (growth yield during feast phase);
[h]X_{max} (maximum active biomass concentration);
[i]$PHA_{end\ test}$ (PHA at the end of the test).

Fermented cheese whey as substrate

The second and the third tests were performed in order to evaluate the ability of selected PHAs-accumulating bacteria to produce PHA by using cheese whey as the carbon source (FCW1 and FCW2 for the second and third test respectively).

In particular, the second test (FCW1) showed an F/F ratio trend that was in the correct range (0.1-0.3), indicating that a good selection in PHAs-accumulating bacteria had occurred[45]. The maximum PHA content at the end of the feast phase was of 296 g kg VSS^{-1} (Table 2.3, Selection Process), with the PHA being composed exclusively by PHB. The specific organic acids consumption rate, the specific PHA storing rate and the specific growth rate were of 0.4 mmole OA-C mmole X-C^{-1} h^{-1}, 0.2 mmole PHA-C mmole X-C^{-1} h^{-1} and 0.08 mmole X-C mmole X-C^{-1} h^{-1}, respectively. The storage yield was equal to 0.7 and the growth yield was lower than the storage one, and it was equal to 0.23.

During the second test, an accumulation assay was carried out in order to evaluate the maximum PHA accumulating capacity of the selected culture produced with the FCW1, using the same substrate as carbon source. The trend of the accumulation assay is reported in figure 2.1(b).

As reported in table 2.3 (Accumulation Process), the PHA at the end of the test was equal to 659 g kg VSS^{-1} and it was composed only by PHB. The storage yield was of 0.7 mmole PHA-C mmole OA-C^{-1} and the PHA productivity was equal to 10.9 g PHA L^{-1} d^{-1}.

For the third test, 1 L working volume SBR containing the culture selected with FCW1 was used and fed with FCW2. The test showed that although the culture was submitted to a substrate change, the F/F ratio remained within the range to be considered correct to keep the culture well selected in terms of PHA-accumulating bacteria.

When the culture was well enriched in PHA-storing microorganisms, the maximum PHA at the end of the feast phase was 390 g kg VSS^{-1} (Table 2.3, Selection Process); both HB and HV composed the polymer accumulated, in the percentage of 59 % 3-hydroxybutyrate and 41 % 3-hydroxyvalerate, respectively.

The specific organic acids consumption rate, the specific PHA storing rate and the specific growth rate were of 0.5 mmole OA-C mmole X-C^{-1} h^{-1}, 0.4 mmole PHA-C mmole X-C^{-1} h^{-1} and 0.06 mmole X-C mmole X-C^{-1} h^{-1}, respectively. The storage yield was equal to 0.9 and the growth yield was equal to 0.1.

During the third test, an accumulation assay was conducted in order to evaluate the maximum PHA storing capacity of the culture maintained with the FCW2 (Figure 2.1c). As reported in table 2.3 (Accumulation Process), the PHA at the end of the test was equal to 814 g kg VSS^{-1}, and it was composed of both HB and HV in the percentages of 60 % 3-hydroxybutyrate and 40 % 3-hydroxyvalerate, respectively. The storage yield was of 0.8 mmole PHA-C mmole OA-C^{-1} and the PHA productivity was equal to 28.2 g PHA L^{-1} d^{-1}.

Table 2.3 *Parameters characterizing the selection process and the accumulation assay using both fermented cheese whey 1 and fermented cheese whey 2*

Selection Process

Substrate	**F/F**[a] ($h\ h^{-1}$)	**PHA_{max}**[b] (g PHA kg VSS^{-1})	**Polymer composition (ΔHB:ΔHV)** (%, W/W)	**$-q_{OA}$**[c] (mmole OA-C mmole X-C^{-1} h^{-1})	**q_{PHA}**[d] (mmole PHA-C mmole X-C^{-1} h^{-1})	**q_X**[e] (mmole X-C mmole X-C^{-1} h^{-1})	**$Y_{PHA/OA}$**[f] (mmole PHA-C mmole OA-C^{-1})	**$Y_{X/OA}$**[g] (mmole X-C mmole OA-C^{-1})	**X_{max}**[h] (g VSS L^{-1})
FCW1	0.1±0.0	296±21	100:0	0.4±0.0	0.2±0.0	0.08±0.01	0.7±0.0	0.23±0.0	2.7±0.2
FCW2	0.1±0.0	390±27	59:41	0.5±0.0	0.4±0.0	0.06±0.01	0.9±0.1	0.1±0.0	2.2±0.1

Accumulation Process

Biomass selected with:	**Substrate**	**Test length** (h)	**$PHA_{end\ test}$**[i] (g PHA kg VSS^{-1})	**Polymer composition (ΔHB:ΔHV)** (%, W/W)	**$-q_{OA}$** (mmole OA-C mmole X-C^{-1} h^{-1})	**q_{PHA}** (mmole PHA-C mmole X-C^{-1} h^{-1})	**$Y_{PHA/OA}$** (mmole PHA-C mmole OA-C^{-1})	**PHA Productivity** (g PHA L^{-1} d^{-1})
FCW1	FCW1	5.1±0.1	659±46	100:0	0.3±0.0	0.2±0.0	0.7±0.0	10.9±0.8
FCW2	FCW2	2.1±0.0	814±57	60:40	0.5±0.1	0.4±0.0	0.8±0.1	28.2±2

[a] F/F (feast to famine ratio);
[b] PHA_{max} (PHA at the end of the feast phase);
[c] $-q_{OA}$ (specific organic acids uptake rate);
[d] q_{PHA} (specific PHA accumulation rate);
[e] q_X (specific growing rate);
[f] $Y_{PHA/OA}$ (storage yield);
[g] $Y_{X/OA}$ (growth yield during feast phase);
[h] X_{max} (maximum active biomass concentration);
[i] $PHA_{end\ test}$ (PHA at the end of the test).

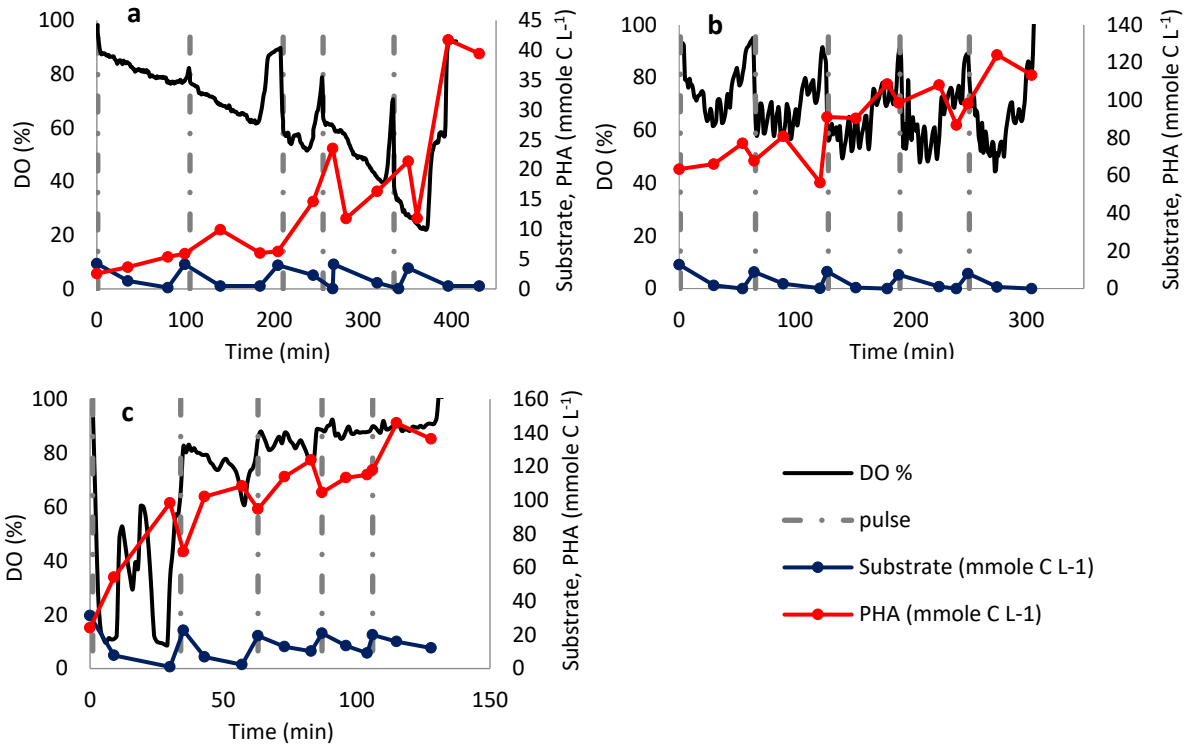

Figure 2.1 *DO trend (%), substrate consumption trend (mmole C L^{-1}) and PHA accumulation trend (mmole C L^{-1}) during the accumulation assays. (a) Accumulation test with sodium acetate; (b) accumulation test with fermented cheese whey 1; (c) accumulation test with fermented cheese whey 2*

PHA production and quality: a comparison approach

Results obtained in this study indicated that PHA can be produced by fermented cheese whey, giving very good yields (Y_{tot}, mg COD_{PHA} mg COD_{SS}^{-1}), that were in line with those reported in the literature for both cheese whey and other organic substrates (Table 2.4).

Data obtained indicated that the second process (FCW1 as substrate) had a lower PHA yield (Y_{tot} of 0.24 mg COD_{PHA} mg COD_{SS}^{-1}) than that obtained in the third process (FCW2 as substrate) (Y_{tot} of 0.42 mg COD_{PHA} mg COD_{SS}^{-1}). The total process yield (Y_{tot}) was the consequence of the soluble COD fermentation to organic acid ($Y_{fermentation}$) and of the successive acids transformation into PHA ($Y_{storage}$), and because the storage yields for the two processes were quite similar (Table 2.4), differences were due probably to the different organic acid yield during the acidic fermentation step. Therefore, results of this work seem to indicate that the fermentation step played an important role in determining the subsequent transformation of soluble COD into PHA. In particular, these results indicated that cheese whey fermented by autochthonous bacteria (FCW1) was less effective in producing organic acids than fermentation

performed by using heat-shocked digestate-inoculum (FCW2), and that the fermentation process acted as limiting factor for the successive transformation of soluble COD into PHA. Taking into consideration data of this work in comparison with those from the literature (considering only cheese whey substrate) (Table 2.4) a very good linear correlation was found for total yield (Y_{tot}) *vs.* fermentation yield ($Y_{fermentation}$) (r= 0.90; $p< 0.05$; n= 4).

In terms of PHA accumulation in cells ($PHA_{end\ test}$, g PHA kg VSS^{-1}), results obtained in this work were in line with or much better than those reported in the literature (Table 2.4). The best result was obtained when the FCW2 was used as the substrate for the mixed microbial culture (Table 2.4). This result was due probably to the fact that PHA accumulation was performed using biomass previously selected during tests that used the FCW1 (see M & M), allowing the culture in this phase of biomass to become more enriched in PHAs-accumulating bacteria. The presence of an enriched PHAs-accumulating biomass was also confirmed by the data reported in Table 2.3 and concerning the two accumulation processes. In particular the specific organic acids uptake rate, the specific PHA storing rate and the storage yield in the test with FCW1 were, respectively, of 40 %, 50 % and 13 % lower than those obtained in the test with FCW2, indicating a lower effectiveness of the MMC in PHA production.

Accumulation data obtained in this work ($PHA_{end\ test}$) were much better than those reported by Carletto[102], who did not use fermented cheese whey, but commercial cheese whey powder and permeated cheese whey (Table 2.4). The data are in line with those reported by Duque et al.[66] that used a pre-fermented chees whey. Nevertheless, in this work data obtained were related to two different fermentations performed to obtain two feedstocks characterized by two different acid compositions, i.e. one more rich in lactic acid and one mainly composed by volatile fatty acids.

It is well documented that feeding mixed microbial culture with sugars can lead to microbial media enriched in other bacteria than just PHAs-storing ones (i.e. glycogen-storing bacteria), reducing final PHA content in the biomass.[53,108] These data confirm that the best substrates to produce PHA from mixed microbial culture are those rich in organic acids that are direct precursors of PHA.[43,53]

From a qualitative point of view, the use of fermented cheese whey gave two different polymers: FCW 1 produced 100 % polyhydroxybutyrate (PHB), and FCW2 gave a copolymer composed by both HB (60 %) and 3-hydroxyvalerate (HV) (40 %). The different composition of the two polymers derived from the different composition of the two fermented cheese wheys

used. In particular, the FCW1 contained lactic, butyric and acetic acids that are all precursors of HB.[66,109] Carletto[102] by using commercial cheese whey powder and a permeated cheese whey (Table 2.4) also obtained pure PHB. In that case, the substrates were formed by disaccharide lactose that after hydrolysis, giving glucose and galactose, produces pyruvate by the glycolysis pathway. This molecule is then transformed into acetyl-CoA and successively by the PHB-anabolic pathway into PHB.[43,110]

The FCW2 was composed by acetic, propionic, butyric, lactic and valeric acids. In this case, the presence in the substrate of HV precursors, i.e. propionic and valeric acids[66,111] led to the production of a polymer composed, also, of 40 % HV (Table 2.4).

Table 2.4 *Comparison among cheese whey and other agro-industrial wastes used as substrate for PHAs production from mixed microbial cultures*

PHAs production from MMC using cheese whey and other agro-industrial wastes							
Substrate	**$PHA_{end\ test}$[a]** ($g\ PHA\ kg\ VSS^{-1}$)	**Polymer composition (ΔHB:ΔHV)** (%, W/W)	**$Y_{fermentation}$[b]** ($mg\ COD_{OA}\ mg\ COD_{SS}^{-1}$)	**$Y_{storage}$[c]** ($mg\ COD_{PHA}\ mg\ COD_{OA}^{-1}$)	**Y_{tot}[d]** ($mg\ COD_{PHA}\ mg\ COD_{SS}^{-1}$)	**PHA Productivity** ($g\ PHA\ L^{-1}\ d^{-1}$)	**Ref.**
Fermented cheese whey 1	659±46	100:0	0.4±0.0	0.6±0.0	0.24±0.02	10.9±0.8	This study
Fermented cheese whey 2	814±57	60:40	0.6±0.1	0.7±0.1	0.42±0.03	28.2±2	This study
Molkolac®	517	100:0	-	-	-	0.1	[102]
Permeated cheese whey	375	100:0	-	-	-	0.4	[102]
Fermented cheese whey	650	81:19	0.74±0.18	0.67±0.13	0.5	13.4	[66]
Fermented cheese whey	-	from 83:17 to 85:15	0.5	0.4	0.2	-	[67]
Fermented olive oil mill effluents	540	89:11	0.36 ($mg\ COD_{OA}\ mg\ COD_{Stot}^{-1}$)	1	0.36 ($mg\ COD_{PHA}\ mg\ COD_{Stot}^{-1}$)	-	[73]
Fermented molasses	560	48:52	0.80±0.19	0.85±0.19	0.68	8.88	[66]
Fermented candy bar factory wastewaters	760	84:16	0.64±0.15	-	0.3±0.04	12	[71]

[a] $PHA_{end\ test}$ (PHA at the end of the test);
[b] $Y_{fermentation}$ (conversion yield from soluble COD to organic acids);
[c] $Y_{storage}$ (conversion yield from organic acids to PHA);
[d] Y_{tot} (conversion yield from soluble COD of the initial substrate to PHA).

In conclusion, the results of this work indicate that by modifying the organic acid composition of the fermented cheese whey it was possible to modify polymer composition, in agreement with the literature[43,66,106]. Now taking into consideration the PHA composition (HV presence)

for the polymers obtained in this and other works (Table 2.4), and the presence of HV-precursors in the fermented media, i.e. valeric plus propionic acids (Table 2.5), a very good correlation between these two parameters was found (r= 0.96; $p< 0.01$; n= 8).

Hydroxyvalerate in the PHA polymer, reduces the crystallinity, decreases stiffness and brittleness of the material and increases the flexibility, widening the processing window for the material.[43,112] The possibility to predict PHA composition taking into consideration organic acid composition in the fermented substrate could represent an interesting result, showing that it is possible to modulate acid fermentation (and so acid composition of the fermented slurry) by using different inocula.

Future full-scale applications of the results obtained need to take into account PHA yield from the starting substrate (e.g. cheese whey). PHA accumulation tests performed in this work with FCW1 and FCW2 gave 60 g PHA $kg_{cheese\ whey\ 1}$ TS^{-1} and 70 g PHA $kg_{cheese\ whey\ 2}$ TS^{-1}, respectively. Unfortunately, not many data about total PHA yield by using MMC are reported in the literature and the only one refers to the use of fermented food waste as substrate[78]. In that case a PHA yield of 30 g PHA $kg_{food\ waste}$ TS^{-1} was reported, this being much lower than the PHA yields obtained in this work, although a direct comparison is difficult because of the different nature of the substrates considered.

Table 2.5 *Relation between the presence of HV in the polymer and the presence of propionate and valerate in the substrate fed using cheese whey and other agro-industrial wastes for PHAs production from mixed microbial culture*

Substrates	**HV in PHA** (%, W/W)	**Propionate** (%, COD_{OA} basis)	**Valerate** (%, COD_{OA} basis)	**Ref.**
Fermented cheese whey 1	0	0	0	This study
Molkolac®	0	0	0	[102]
Permeated cheese whey	0	0	0	[102]
Fermented olive oil mill effluents	11	9.9	2.1	[73]
Fermented candy bar factory wastewaters	16	9	3.2	[71]
Fermented cheese whey	19	6	4	[66]
Fermented cheese whey 2	40	19	4	This study
Fermented molasses	52	35	13	[66]

2.4 Conclusions

The results of this work indicate that fermented cheese whey can be used producing PHA starting from mixed microbial culture. Cheese whey fermentation plays an important role as it produces PHA precursors and, depending of the kind of organic acid produced, modulates PHA composition.

Total PHA yields obtained in this work were encouraging in view of the possible future reuse of cheese whey to produce PHA.

Acknowledgment

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3. Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture

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Abstract

Background

In Europe, almost 87.6 million tonnes of food waste are produced. Despite the high biological value of food waste, traditional management solutions do not consider it as a precious resource. Many studies have reported the use of food waste for the production of high added value molecules. Polyhydroxyalkanoates (PHAs) represent a class of interesting bio-polyesters accumulated by different bacterial cells, and has been proposed for production from the Organic Fraction of Municipal Solid Waste (OFMSW). Nevertheless, until now, no attention has been paid to the entire biological process leading to the transformation of food waste to organic acids (OA) and then to PHA, getting high PHA yield per food waste unit. In particular, the acid-generating process need to be optimized, maximizing OA production from OFMSW. To do so, a pilot-scale Anaerobic Percolation Biocell Reactor (100 L in volume) was used to produce an OA-rich percolate from OFMSW which was used subsequently to produce PHA.

Results

The optimized acidogenic process resulted in an OA production of 151 g kg^{-1} from fresh OFMSW. The subsequent optimization of PHA production from OA gave a PHA production, on average, of 223 ± 28 g kg^{-1} total OA fed. Total mass balance indicated, for the best case studied, a PHA production per OFMSW weight unit of 33.22 ± 4.2 g kg^{-1} from fresh OFMSW, corresponding to 114.4 ± 14.5 g kg^{-1} of total solids from OFMSW. PHA composition revealed a hydroxybutyrate / hydroxyvalerate (%) ratio of 53 / 47 and Mw of $8 \cdot 10^5$ kDa with a low polydispersity index, i.e. 1.4.

Conclusions

This work showed how by optimizing acidic fermentation it could be possible to get a large amount of OA from OFMSW to be then transformed into PHA. This step is important as it

greatly affects the total final PHA yield. Data obtained in this work can be useful as the starting point for considering the economic feasibility of PHA production from OFMSW by using mixed cultures.

3.1 Background

Food Waste (FW) is defined as the organic material produced for human consumption and discarded, lost or degraded, primarily at manufacturing, retail and consumption stages.[16] In Europe, almost 87.6 million tonnes of FW are produced annually.[17] These wastes are characterised by both high moisture and high biodegradability, so FW creates adverse environmental impacts if landfilled (odours, fires, volatile organic compounds, groundwater contamination by leachate, global climate changes, etc.) and also, high disposal costs (88-144 \$ tonne^{-1}).[16] European and national legislation have focused on avoiding FW landfilling by treating it through a thermal process (incineration) or, more frequently, by biological processes (anaerobic digestion and composting) to be carried out on the separately collected Organic Fraction of Municipal Solid Waste (OFMSW).[18] Despite the high biological value of FW, traditional management solutions do not consider it as a precious resource, adding only a small amount of value to the final product i.e. 60-150 \$ tonne^{-1} of biomass for electricity production and 5-10 \$ tonne^{-1} of biomass for compost production.[19] Therefore supporting these activities generally requires government contributions or high tariffs for citizens, e.g. in Italy the waste tariff increased by 70 % in the last 10 years.

FW is an organic matrix rich in valuable molecules such as starch, cellulose, hemicellulose, lignin, proteins, lipids and organic acids that could be managed in a more sustainably economic way by using it as a raw material for bulk chemicals production.[19] Many studies have reported the use of FW for the production of high added value molecules such as lactic acid, citric acid, succinic acid, single cell oils, enzymes and polymers,[20] an approach which is more financially rewarding in comparison with compost and biomethane production (1,000 \$ tonne^{-1} of biomass for bulk chemicals production from FW)[21].

Among the wide varieties of bio-products obtainable from FW are the polyhydroxyalkanoates (PHAs), a class of interesting bio-polyesters accumulated by different bacterial cells under the form of granules inside the cytoplasm. PHAs are completely biodegradable and are mainly produced starting from renewable sources; their chief interesting property is their mechanical behaviour that make them comparable to common plastics.[43] Microbial PHA production can be carried out by using either pure or mixed microbial cultures (MMCs). MMCs with high PHA

accumulation capacity have been suggested as a solution for reducing the high maintenance costs of pure cultures. Many studies related to PHA production by MMC have performed both selection of PHA-storing cultures and PHA accumulation by using just OA as substrates, since they are the direct metabolic precursors of PHA,[43] while PHA production carried out by using pure cultures allows the employment of both OA and simple sugars as carbon sources. Working with mixed microbial cultures, simple sugars are not recommended as they can be used by the culture for the production of other molecules (i.e. glycogen), reducing the final PHA production yield.[53,108] Given the high costs of synthetic OA, agro-industrial wastes, such as molasses, cheese whey, olive oil mill effluent, palm oil mill effluent, candy bar factory wastewater and many other waste streams,[66,71,73,76,113] can be treated by acidic fermentation to produce OA which can then be transformed into PHA by MMC.

Complex wastes, such as OFMSW, have been reported in the literature to be used as substrates for PHA production by MMC.[78,79,80,114] These studies focused attention on PHA production from OA obtained by the fermentation of OFMSW, but until now, no attention has been paid to the entire process leading to the transformation of OFMSW to OA and then to PHA, i.e. to obtain overall estimates of PHA yield based on OFMSW weight unit used. These data, together with those showing the scientific feasibility in transforming organic waste into PHA, are important to estimate the effective economic sustainability of PHA production from OFMSW. In this work, a two-stage approach, i.e. OA production from OFMSW and subsequent PHA biosynthesis from OA, is proposed.

Optimization of the acidic fermentation of OFMSW was carried out in order to exploit the biological potential of this complex matrix, obtaining a percolate rich in OA used subsequently as the substrate to produce PHA by employing MMC. Global mass balance of the process performed was achieved as well as the complete characterization of the polymer obtained.

3.2 Methods

Percolates production from OFMSW

Organic Fraction of Municipal Solid Waste collection

The source-separated OFMSW coming from street bin containers was collected at a full scale composting plant located in northern Italy. Collection was done by following the quartering method: in brief, the OFMSW was sampled at different points of the whole mass (500 kg wet weight – w.w. – x 3 times); then the collected waste was mixed, quartered and sub-sampled until a final sample of about 300 kg w.w. material was reached. OFMSW was stored at 4 °C

before the trials were set up. A representative sample of approximately 30 kg, obtained by mixing sub-samples of about 5 kg each taken randomly from sampled material, was dried and crushed to 2 mm and then used to perform analytical analyses.

The bulking agent (shredded wood) from the same plant was also collected following a similar approach.

Liquid digestate, used to irrigate OFMSW during dry anaerobic fermentation (see later), was collected directly from a Continuously Stirred Tank Reactor Anaerobic Digester (CSTR-AD) plant fed with OFMSW, located in northern Italy and previously described.[115] Digestate was collected from this plant in large quantities from the discharge pipe system of the post digester after 30 days of retention time (HRT).

Experimental apparatus

The acidogenesis (SSAD) trials were carried out by developing a pilot scale Anaerobic Percolation Biocell Reactor (APBR) that was made up of an insulated vertical cylinder (100 L of volume) of PVC material with a hermetic cap. Inside the reactor there was a stainless steel basket with holes at the bottom, into which OFMSW was introduced from the APBR cap, as well as the digestate used to irrigate the organic wastes (Fig. 3.1).

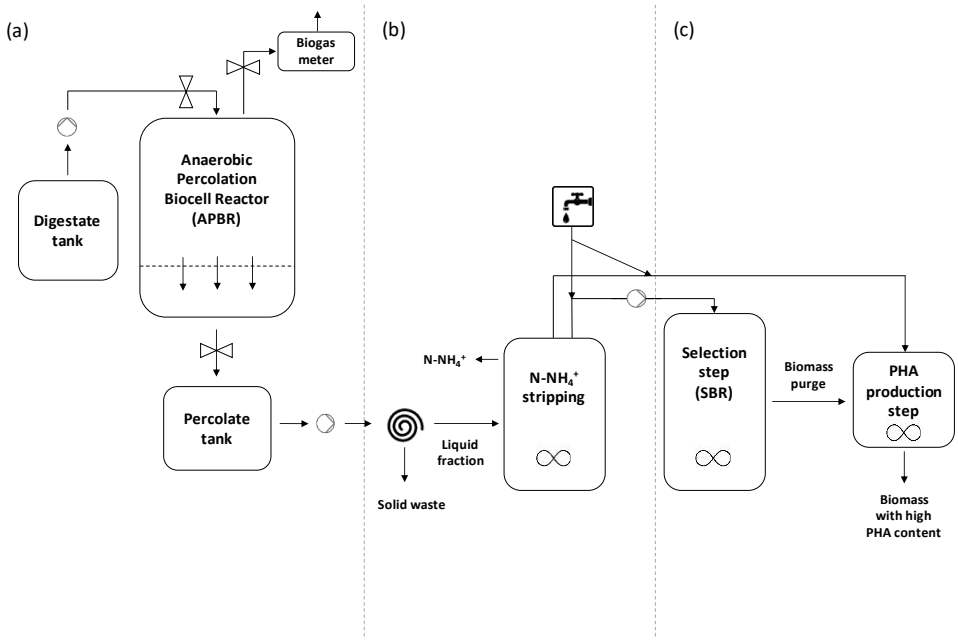

Figure 3.1 *Scheme of the two-stage process used to produce PHA: OFMSW fermentation producing OA (a), percolate pre-treatments (b) and PHA production (c)*

From the bottom of the APBR, the percolate daily generated because of digestate irrigation was extracted and weighed. Digestate irrigation was used to buffer the acidity produced during OFMSW fermentation and to remove OA produced in the APBR. This encouraged high hydrolysis performance from the organic waste, which is the limiting step in producing large amounts of OA from organic substrates.[116,117] Moreover, large-scale OA production becomes toxic for methanogenic bacteria, limiting methane production during the trials.

Experimental setup

Three different percolation trials were carried out. For all of them fresh OFMSW (85 % w.w.) and bulking agent (chopped green waste material) (15 % w.w.) were mixed just before reactor filling to avoid clogging problems in the APBR, allowing the percolation. For each trial, the APBR was filled with about 50 kg of mixture (OFMSWmix-in).

The OFMSWmix-in in the APBR was irrigated continuously by using the liquid digestate obtained from the full-scale CSTR-AD plant in order to optimize OFMSW hydrolysis and subsequent acidogenesis fermentation to produce OA. Anaerobic hydrolysis and acidic fermentation lasted for 21 days for each trial performed. During this time, irrigation with the liquid digestate (previously heated to 55 °C) was done continuously by using a peristaltic pump, adopting an OFMSWmix-in/digestate ratio of 1:0.45 kg kg^{-1} (flow rate of 0.02 L h^{-1} kg^{-1} $OFMSW_{w.w.}$) for the first trial (Trial 1), 1:0.9 kg kg^{-1} (flow rate of 0.04 L h^{-1} kg^{-1} $OFMSW_{w.w.}$) for the second trial (Trial 2), and of 1:1.8 kg kg^{-1} (flow rate of 0.08 L h^{-1} kg^{-1} $OFMSW_{w.w.}$) (day 1 to day 8) and of 1:0.9 kg kg^{-1} (flow rate of 0.04 L h^{-1} kg^{-1} $OFMSW_{w.w.}$) (day 9 to day 21) for the third trial (Trial 3), respectively. The daily-generated percolates were collected from the bottom of the APBR, quantified and mixed together to get a final mixed percolate sample for each trial.

Percolates treatment before PHA production

The three percolates obtained from anaerobic acidic fermentation trials were submitted to two pre-treatments before their use for polyhydroxyalkanoate (PHA) production. The first pre-treatment was to centrifuge the percolates at 20,000 g for 15 minutes, reducing suspended organic carbon from percolates. Supernatants obtained underwent a second pre-treatment adopted to remove ammonia by stripping. This step was performed by bringing supernatant-pH to 11 by adding 6-mole L^{-1} KOH and stirring it (magnetic stirring at 200 rpm) under the hood until a final C:N close to 10 was reached[113]. Then ammonia stripping was stopped and the pH was brought again to the initial value of 8 by using 3 mole L^{-1} H_2SO_4. Ammonia stripped was

not trapped at lab scale and it was not considered in this work. Obviously, this step needs to be considered and further studied on a larger scale.

After pre-treatments, the three supernatants were diluted with deionized water, to produce a final COD content close to 1,300 mg L^{-1}.[113] These OFMSW-derived supernatants (OFMSW-supernatants$_{in}$) were used for subsequent inoculum selection.

Substrates (OFMSW-supernatants$_{acc.}$) used for PHA accumulation tests derived from percolates that had undergone the same treatments shown for those destined for the inoculum selection. Three OFMSW-supernatants$_{acc.}$ were obtained but, in this case, the ammonia content was lower than those reported for OFMSW-supernatants$_{in}$, since it has been reported that N starvation can determine a greater conversion of carbon into PHA because of cell growth limitation[57]. Moreover, OFMSW-supernatants$_{acc.}$ were less diluted, giving a final COD concentration of about 7,500 mg L^{-1}, in order to avoid an excessive dilution of the biomass in the accumulation reactor[66].

Inoculum production and PHA-producing bacteria enrichment

The enrichment of PHA-producing bacteria was performed by using an inoculum constituted by an activated sludge (5 g total suspended solids L^{-1}) collected from the secondary sedimentation tank of a wastewater treatment plant (5.2 x 10^5 equivalent inhabitants) located at Peschiera Borromeo (Milan, Italy).

The enrichment in PHA-producing bacteria was carried out in a Sequencing Batch Reactor (SBR) with a working volume of 800 mL, applying an aerobic dynamic feeding (ADF) strategy.[113]In brief the SBR cycle length was of 12 h, consisting of four discrete phases: i. influent filling (4 min), ii. aeration (675 min), iii. settling (40 min), and iv. withdrawal of the exhausted effluent (5 min), with 1 day of Hydraulic Retention Time (HRT) and 5 days of Sludge Retention Time (SRT), keeping the temperature at 25 °C and the pH at 8.8, this latter controlled automatically by adding 1 mole L^{-1} HCl. Aeration and agitation were provided by supplying air at 6 L min^{-1} and stirring set at 110 rpm. Pumping, aeration and stirring were automatically controlled.

The selection process lasted for three months; for each month, a different pre-treated supernatant was used as substrate: OFMSW-supernatant$_{in}$ 1, OFMSW-supernatant$_{in}$ 2 and OFMSW-supernatant$_{in}$ 3, respectively for the first, second and third month of selection.

The selection trend was monitored by determining the duration of the feast phase achievable by using the dissolved oxygen (DO) concentration in the selection media,[66] measured by an optical probe (FDO 925, WTW, Germany).

In particular, the feast (h) to famine (h) ratio (F/F ratio) was calculated as the ratio between the lengths in hours of the two phases. For a correct selection of PHA-storing bacteria the F/F ratio had to be equal or less than 0.33.[45] To carry out the selection of PHA-accumulating bacteria, 400 mL of activated sludge were used as inoculum feed for each cycle, with 400 mL of OFMSW-supernatant$_{in}$. Organic Loading Rate (OLR) was kept close to 1,300 mg COD L^{-1} d^{-1} and the C:N:P ratio was of about 100:13:0.4 mmole C: mmole N: mmole P. Every month two cycles were monitored in order to evaluate the performance of the selected culture. In particular, monitoring was performed between cycles 31 and cycle 45 since it was reported that the cultures reach stability after three SRTs from the beginning of the trial (cycle 31).[106]

PHA accumulation

The ability of the MMC to accumulate PHA was assessed by fed-batch assays carried out in a 300 mL working volume glass reactor, with continuous aeration and stirring. These assays consisted of feeding the substrate to 160 mL of enriched culture (at least 3 SRTs from the beginning of the selection)[106] adopting a pulse-wise feeding method. The assays were monitored continuously by measuring the concentration of the dissolved oxygen in the accumulation media.[66] In particular, substrate (OFMSW-supernatant$_{acc.}$) was fed to the reactor when DO showed a strong increase.[66] Total C dosed was calculated taking into account that the ratio of the carbon to the microorganisms had to be the same as that inside the selection reactor. The assays were stopped when no DO variation followed the substrate feeding.

For the accumulation tests, the operating conditions used were those adopted in the selection reactor, i.e. temperature of 25 °C, aeration of 6 L min^{-1} and stirring at 110 rpm.

The biomass from the selection process was submitted to accumulation tests using the same substrate as the carbon source; every month two accumulation trials were performed in duplicate, between the cycle 31 and 45.

PHA extraction

The biomass collected after PHA accumulation tests was centrifuged at 8,000 g for 15 min, washed with 0.9 % sodium chloride solution and centrifuged again at 8,000 g for 20 min. The pellet obtained was lyophilized and then suspended in chloroform (in a ratio of ca. 40 mL $CHCl_3$ g^{-1} dried cells) and left to dissolve for a period of 3 days at 37 °C.[66] The solution was then

filtered to remove all undissolved material. The extracted PHA in chloroform was then precipitated by the addition of 5 volumes of methanol and allowed to settle down for 30 min.[118] The white precipitate formed was then filtered, suspended in chloroform and used to fill glass Petri dishes. Finally, chloroform was evaporated allowing polymer recovery in the form of a thin film.

Analytical procedures

OFMSW, digestate and percolates characterization

Total solids (TS), volatile solids (VS), total nitrogen (TKN), pH and ammonia ($N\text{-}NH_4^+$) (detected on fresh material) contents were determined according to the standard procedures[119]. Total OA expressed as acetic acid, were detected on fresh material and determined according to the acid titration method[120].

Substrate and biomass characterization during PHA production

The substrates (OFMSW-supernatants) fed during the selection and accumulation processes were characterized in terms of pH, TS, VS, Chemical Oxygen Demand (COD), OA content (acetate, butyrate, lactate, propionate and valerate), TKN, $N\text{-}NH_4^+$ and phosphorus (P) content. During the selection trials, samples were taken during the cycle once in each SRT; every sample was characterized in terms of total suspended solids (TSS), volatile suspended solids (VSS), soluble COD, OA content, $N\text{-}NH_4^+$ content and PHA content. During accumulation trials, samples were taken continuously in order to measure TSS, VSS, soluble COD, OA content and PHA content. Biomass concentration was calculated as VSS according to the standard methods.[66]

TSS and VSS were determined as reported by Valentino et al.[67]. OA concentrations measured on filtered samples (filter diameter of 0.45 µm) were determined by high performance liquid chromatography (HPLC) using a chromatograph equipped with a UV detector and Aminex HPX-87H column (column temperature 20 °C, 0.0025 M H_2SO_4 eluent, flow rate 0.6 mL min^{-1}). The OA concentrations were calculated through a standard calibration curve (20-1000 mg L^{-1} of each organic acid). The COD and the $N\text{-}NH_4^+$ content (filtered at 0.45 µm) were determined using cuvette test kits (Macherey-Nagel, Germany).

PHA were determined by GC MS using a method adapted from Serafim et al.[57]. Lyophilized biomass was incubated for methanolysis in a 20 % v/v H_2SO_4 in MeOH solution (1 mL) and extracted with chloroform (1 mL). The mixture was digested at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was extracted

and injected (1 mL) into a gas chromatograph equipped with a detector (7980, Agilent Technologies, USA) and a ZB-Wax column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness, Zebron, Phenomenex, USA), using helium as carrier gas at 1.0 mL min^{-1}. Samples were analysed under a temperature regime starting at 40 °C, increasing to 100 °C at a rate of 20 °C min^{-1}, to 175 °C at a rate of 38 °C min^{-1} and reaching a final temperature of 220 °C at a rate of 20 °C min^{-1} for ensuring cleaning of the column after each injection. Injector and detector temperatures were at 280 °C and 230 °C, respectively. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through the use of two calibration curves, one for HB and another for HV, using standards (0.1-8 g L^{-1}) of a commercial P(HB-HV) (88 %/12 %) (Sigma Aldrich, Germany), and corrected using heptadecane as internal standard (concentration of approximately 1 g L^{-1}) (Sigma Aldrich, Germany).

PHA and active biomass growth yield calculation

The PHA content in cells was referred to VSS on a mass basis [PHA= (g kg^{-1} VSS)], considering VSS to be constituted by both active biomass (X) and PHA.[66] PHA was converted into COD according to the following oxidation stoichiometry: 1.67 mg COD mg^{-1} HB monomer and 1.92 mg COD mg^{-1} HV monomer.[45]

Acetate, butyrate and lactate were considered as HB precursors, valerate and propionate as HV precursors.[66] X was calculated on a COD basis considering that 1 g of X contains 1.42 g of COD.[45] For the SBR, PHA storage yield was expressed in COD and referred to both COD consumed ($COD_{cons.}$) and OA consumed ($COD_{OA\text{-}cons.}$), calculated respectively as the ratio between the amount of PHA accumulated during the feast phase (COD_{PHA}) and the amount of COD depleted or OA depleted.

In the accumulation batches, these PHA storage yields were calculated as described before, for each pulse. In order to compare different accumulation tests, the average values of the first three pulses and for each parameter were considered.[45]

In accumulation batches PHA storage yield was also related to COD fed (COD_{in}) and OA fed (COD_{OAin}), moreover it was also related back to total solids of OFMSW ($OFMSW_{TS}$) and to $OFMSW_{w.w.}$.

The X growth yield in the SBR was expressed in COD and referred to COD consumed ($COD_{cons.}$), calculated as the ratio between the new X produced during the feast phase (COD_X) and the amount of COD depleted.[45]

PHA characterization by solid state NMR and solution ^{1}H NMR, and molecular weight

Solid-state ^{13}C NMR spectra of lyophilized biomass containing PHA were recorded on a Bruker Avance 300 spectrometer operating at 75.47 MHz, using a 4x21 mm cylindrical zirconium rotor spun at 11000 Hz to avoid the side bands. The ^{13}C cross polarization magic angle spinning (CPMAS) NMR spectra were acquired using recycle delay of 8 s, ^{1}H 90 pulse length of 3.5 μs, 1 m contact time, acquisition time of 35 ms and from 1K to 4K scans. The ^{13}C single pulse excitation (SPE) NMR spectra were recorded with delays of 160 s and 1K-2K scans.

The chemical shifts were recorded relative to tetramethylsilane via benzene as a secondary reference.

Liquid NMR experiments on extracted PHA were performed on a Bruker 500 MHz AVANCE III NMR spectrometer (Bruker GmbH, Germany) with a 5 mm TCI cryoprobe. Deuterated chloroform (99.96 %, Sigma Aldrich) was used as solvent. ^{1}H NMR spectra were recorded at 303K using recycle delay of 12 s, 64K fid size and 64 scans.

PHA molecular weight was determined by HP-SEC/TDA measurement. The HPLC equipment consisted of a Viscotek system (Malvern Instrument Ltd, Malvern, UK) equipped with a Knauer HPLC pump K501, and a Biotech Degasi GPC degassing device. The detector system was a Viscotek mod. 302 Triple Detector Array (TDA), which is composed by Laser Light Scattering detector (90° and 7°; wavelength 670 nm), Refractive index (RI) detector (cell volume of 12 μL; light emitting diode (LED) at 660 nm wavelength) and Viscosimeter detector (four capillaries with a differential Wheatstone bridge configuration). A PL GEL 20 um MIXED A column (7.5 x 300 mm) was used. Chloroform was used as the mobile phase at a flow rate of 1 mL min^{-1}. Columns, injector and detectors were maintained at 30 °C. Samples were dissolved in chloroform at concentrations of 2-8 mg mL^{-1} and filtered on a 0.2 μm membrane before injection. Injection volume was of 100 μl.

The system was calibrated with the PS narrow standard of known Mw, polydispersity and intrinsic viscosity (Malvern PolyCAL PS std 105k). Using a standard PHA sample at different concentrations (2.3, 4.3, 6.0, 8.5 mg mL^{-1}), the differential refractive index increment (dn/dc) value was found to be equal to 0.024 and used for further calculations.

Statistical analysis

Average and standard deviation values were calculated according to standard procedures and the results were analysed by an ANOVA test. A Tukey test was used to compare mean values and to assess the significance of the differences between mean values, adopting a fixed effects

model, i.e. the digestate flow adopted modified both the chemical composition of the percolates produced and of supernatants derived.

All statistical analyses were carried out using the SPSS statistical software, version 15.0 (SPSS, Chicago,IL).

3.3 Results

Percolation trials

Percolation trials carried out by adopting different irrigation regimes, i.e. varying digestate flow-rate, showed differences in terms of total percolate produced, i.e. an increase of the hourly digestate flow-rate determined an increase of the total percolate produced (Table 3.1). Again, the production of total OA increased by more than 200 % when digestate flow rate doubled, when comparing Trial 1 with Trial 2 (Table 3.1). However, the further increase of digestate flow rate (Trial 3) did not lead to any increase of OA production and on the contrary, slightly decreased it.

Percolates obtained were similar in terms of chemical characteristics apart from the percolate coming from the third trial that appeared more diluted as regarded OA and $N\text{-}NH_4^+$ content (Table 3.1).

Trial 2 optimized OFMSW hydrolysis and subsequent acidogenesis fermentation, producing the highest amount of OA.

Table 3.1 *Percolation trials conditions (a) and composition of OFMSWs and digestates used and of percolates produced (b)*

a

Trial	T (°C)	Duration (day)	OFMSW (kg)	Digestate flow rate (L h^{-1} kg^{-1} $OFMSW_{w.w.}$)	Percolate produced (L kg^{-1} $OFMSW_{w.w.}$)	OA produced [a] (g kg^{-1} $OFMSW_{w.w.}$)
Trial 1	30	21	44	0.02	8.03	60.5
Trial 2	30	21	42.7	0.04	19.3	151
Trial 3	40 (day 1-8) 30 (day 9-21)	21	43.6	0.08 (1-8 day) 0.04 (9-21 day)	25.57	139

b

Trial		TS (%)	VS (%)	pH	OA[a] (mg L^{-1})	$N\text{-}NH_4^+$ (mg L^{-1})
Trial 1,2,3	**OFMSW (average)**	24.5±3.9	86.8±2.6	4.3 ± 0	11,663±1.649	304±239
Trial 1,2,3	**Digestate (average)**	2.2±0.2	70.8±2.7	8.32±0.1	3,448±1.591	2,065±343
Trial 1	**Percolate 1 (P1)**	2.4±0.49a[b]	71.2±2.2b	6.91±0.19a	7,540±1,474b	2,231±130b
Trial 2	**Percolate 2 (P2)**	1.9±1.1a	66.5±2.8a	6.99±0.61a	7,831±1,775b	2,412±472b
Trial 3	**Percolate 3 (P3)**	2±0a	73±2a	6.99±0.32a	5,437±818a	1,784±117a

[a]OA expressed as acetic acid, determined according to the acid titration method[120];
[b]Limited to percolates, values in the same column followed by the same letter are not statistically different (ANOVA Tukey test, $p<0.05$).

OFMSW-supernatants

Pre-treated percolates used for MMC production (OFMSW-supernatants$_{in}$) showed no statistical differences in terms of COD and N-NH_4^+ contents, apart from the P content and HB/HV precursors ratio which were higher for OFMSW-supernatant$_{in}$ 3 than for the other OFMSW-supernatants$_{in}$ (Table 3.2).

OFMSW-supernatants used to perform PHA accumulation tests (OFMSW-supernatants$_{acc.}$) were again quite similar to each other, with the exception for P content and HB/HV precursors ratio which were higher for OFMSW-supernatant$_{acc.}$ 3 than for the other OFMSW-supernatants$_{acc.}$ (Table 3.2).

Table 3.2 *Chemical composition of the substrates used in the MMC selection and PHA accumulation processes*

Substrate	**COD** (mg L^{-1})	**N-NH_4^+** (mg L^{-1})	**P tot** (mg L^{-1})	**C** (mmole L^{-1})	**N-NH_4^+** (mmole L^{-1})	**P** (mmole L^{-1})	**Organic acids composition (HB precursors/ HV precursors)** (%, weight basis)
OFMSW-supernatant$_{in}$ 1	1,304±10a[a]	46.7±4.9a	3.1±0.3a	34.4±0.3a	2..6±0.3a	0.1±0a	59.7/40.3
OFMSW-supernatant$_{in}$ 2	1,322±37a	56.2±7.5a	3.6±0.4a	34.9±1a	3.1±0.4a	0.12±0.01a	51.2/48.8
OFMSW-supernatant$_{in}$ 3	1,231±63a	58.4±8.1a	5.6±0.6b	32.5±1.7a	3.2±0.5a	0.18±0.02b	67.8/32.2
OFMSW-supernatant$_{acc.}$ 1	7,591±54a	28±4a	16.9±2.3a	200±2a	1.6±0.2a	0.55±0.07a	57.2/42.8
OFMSW-supernatant$_{acc.}$ 2	7,879±87a	29.1±2a	19.8±3a	207.8±2.3a	1.6±0.1a	0.64±0.11a	52/48
OFMSW-supernatant$_{acc.}$ 3	7,253±98a	26.8±3a	31.1±5.2b	191.3±2.6a	1.5±0.2a	1±0b	65.1/34.9

[a]By separating OFMSW-supernatants$_{in}$ from OFMSW-supernatants$_{acc.}$, values in the same column followed by the same letter are not statistically different (ANOVA Tukey test, $p<0.05$).

Mixed microbial culture enrichment

The amount of PHA stored at the end of the feast phase and PHA storage yields, measured for the mixed microbial culture during the selection process, remained quite stable (Table 3.3) during the first two months of selection when OFMSW-supernatants$_{in}$ 1 and 2 were used as substrates. In particular, the PHA stored at the end of the feast phase was subject to small fluctuations with an average value of 363 ± 30 g kg^{-1} VSS. PHA storage yields (expressed as COD) on COD and on OA consumed were, on average, of 0.52 ± 0.07 mg mg^{-1} $COD_{cons.}$, and of 0.86 ± 0.23 mg mg^{-1} $COD_{OA\text{-}cons.}$, respectively, indicating the main role of OA among different carbon sources in PHA production[43,113].

The culture fed with OFMSW-supernatant$_{in}$ 3 gave different results as the polymer content at the end of the feast phase and PHA storage yield on COD consumed were lower by 36 % and 20 % in comparison with the data reported for OFMSW-supernatants$_{in}$ 1 and 2. On the other hand, PHA storage yield on OA consumed increased by 34 %. In any case, performance

obtained with OFMSW-supernatant$_{in}$ 3 was better than that reported in the literature for similar substrates (OFMSW leachate)[79,80] and it was comparable to those obtained with other waste substrates (e.g. fermented molasses and fermented cheese whey)[66,70]. Probably, differences found in terms of reduction of polymer content, were because OFMSW-supernatant$_{in}$ 3 was characterized by a higher presence of P that reduced PHA accumulation[105].

Feast to famine ratio obtained during all selection processes using the three OFMSW-supernatants$_{in}$ (F/F ratio ranged from 0.03 to 0.15) (Fig. 3.2), confirmed the good performance of the selection procedures, indicating successful enrichment in PHA-accumulating bacteria[45].

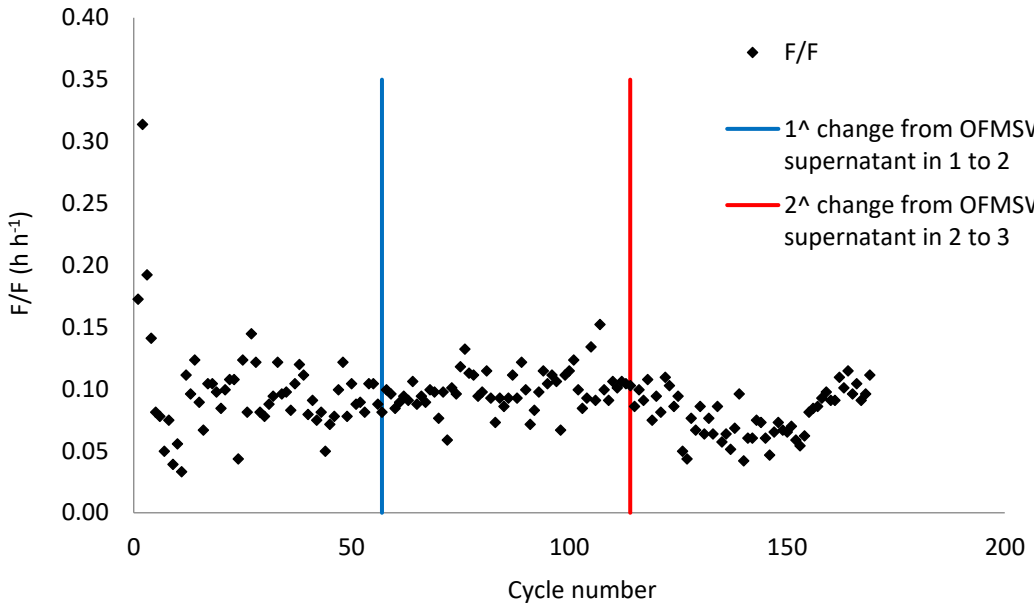

Figure 3.2 *Feast to famine ratio (F/F) during the entire selection process with the three OFMSW-supernatants$_{in}$*

PHA production

Accumulation tests (Table 3.3) revealed a better performance for the cultures selected with OFMSW-supernatants$_{in}$ 1 and 2 than that of 3 in terms of PHA accumulation in relation to VSS. Maximum PHA content at the end of the tests fed with OFMSW-supernatants$_{acc.}$ 1 and 2 was of 467 ± 41 g kg^{-1} VSS and 465 ± 21 g kg^{-1} VSS, respectively, which were about 13 % higher than that obtained in the tests fed with OFMSW-supernatant$_{acc.}$ 3 (Table 3.3). These results were similar to those obtained in previous work using other waste substrates (e.g. palm oil mill effluent, fermented olive oil mill pomace, crude glycerol),[74,100,121] indicating a reliable performance of the cultures in accumulation tests. In terms of PHA storage yields (expressed as COD) on COD consumed, PHA accumulation carried out by using the three supernatants

gave substantially similar results when OFMSW-supernatants$_{acc.}$ 2 and 3 were used, i.e. 0.5 mg mg^{-1} COD$_{cons.}$. These data were higher than those detected for the accumulation trial using OFMSW-supernatant$_{acc.}$ 1.

PHA storage yields (expressed as COD) referred to consumed-OA were very different for different supernatants. For example, PHA yield was reduced from 1.13 to 0.66 mg mg^{-1} COD$_{OA-cons.}$ when comparing the trial performed using the OFMSW-supernatant$_{acc.}$ 1 to the one using the OFMSW-supernatant$_{acc.}$ 3. Residual OA could be potentially reused to produce PHA or destined to the production of biogas and energy, supporting the entire process throughout anaerobic digestion. These solutions have not been considered in this work.

Trials performed using the OFMSW-supernatants$_{acc.}$ 1 and 2, gave the highest PHA production with respect to the total COD and OA fed, i.e., as average, 130 ± 16 g kg^{-1} COD$_{in}$ and 223 ± 28 g kg^{-1} OA$_{in}$, that were 30 % higher than data obtained for the culture fed with the OFMSW-supernatant$_{acc.}$ 3. Lower PHA production was probably due to the decrease of biomass which occurred in the SBR during the last month (data not reported).

PHA composition was constant among the three different accumulation tests performed, with an average value of 56 ± 3 % of HB and 44 ± 3 % of HV, as detected by the GC MS approach. This composition was similar to that obtained by Amulya et al.[79] using fermented OFMSW as substrate.

Table 3.3 *Parameters characterizing the MMC selection and PHA accumulation processes with the three OFMSW-supernatants*

Substrate	**PHA content**[a] ($g\ kg^{-1}$ VSS)	**Polymer composition (ΔHB/ΔHV)** (% w/w)	**PHA Yield**[c] ($mg\ mg^{-1}\ COD_{cons.}$)	**PHA Yield**[d] ($mg\ mg^{-1}\ COD_{OA\text{-}cons.}$)	**PHA Yield**[e] ($g\ kg^{-1}\ COD_{in}$)	**PHA Yield**[f] ($g\ kg^{-1}\ OA_{in}$)	**PHA Productivity** ($g\ L^{-1}\ d^{-1}$)	**X growth Yield**[g] ($mg\ mg^{-1}\ COD_{cons.}$)
Selection								
OFMSW-supernatant$_{in}$ 1	342±23	-	0.51±0.02	0.85±0.26	-	-	-	0.16±0.01
OFMSW-supernatant$_{in}$ 2	383±2	-	0.54±0.07	0.87±0.09	-	-	-	0.05±0.01
OFMSW-supernatant$_{in}$ 3	234±20	-	0.42±0.04	1.15±0.34	-	-	-	0.09±0.01
Accumulation								
OFMSW-supernatant$_{acc.}$ 1	476±41[b]	56.5±3.5/43.5±3.5	0.44±0.17	1.13±0.18	134±22	230±38	4.4±1.3	-
OFMSW-supernatant$_{acc.}$ 2	465±21[b]	54.5±2.6/45.5±2.6	0.52±0.1	0.75±0.21	128±16	220±28	4.3±1.2	-
OFMSW-supernatant$_{acc.}$ 3	405±28[b]	57±2/43±2	0.5±0.1	0.66±0.09	94±5	161±9	7±0.9	-

[a]PHA stored at the end of the feast phase referred to VSS;
[b]PHA accumulated at the end of the accumulation test referred to VSS;
[c]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{cons.}$;
[d]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{OA\text{-}cons.}$;
[e]PHA produced referred to COD_{in};
[f]PHA produced referred to OA_{in};
[g]X growth yield during feast phase expressed as COD (COD_X) referred to $COD_{cons.}$.

3.4 Discussion

PHA production and mass balance

Results above discussed indicated that supernatants obtained worked well as substrates to select and enrich MMC in PHA-storing bacteria. In particular, PHA content at the end of the feast phase and PHA yield on COD and on OA consumed obtained in this work were comparable or higher than those reported in the literature for selection processes of mixed microbial cultures obtained by feeding similar OFMSW leachate[79,80] or other waste substrates (e.g. fermented molasses and fermented cheese whey)[66,70]. Best performances obtained could be due to the fact that in this work culture enrichment was performed at 30 °C, which is an optimal temperature for microbial activity, and because a more balanced C:N ratio (close to 10) was adopted with respect to the lower values adopted by other Authors[78,80].

The good results above discussed allowed us to get PHA contents during PHA accumulation tests higher than those reported in the literature by Amulya et al.[79] and comparable to the data reported by Zhang et al.[114], both working on food wastes (Table 3.4). On the other hand, PHA contents were clearly lower than those obtained by Korkakaki et al.[80], who obtained PHA contents of 778-784 g kg^{-1} VSS (Table 3.4) by feeding fermented food wastes to a MMC, achieving very similar results to those reported for pure cultures (860-870 g kg^{-1} VSS by using *Cupriavidus necator*)[86,87] (Table 3.4). Nevertheless, it should be specified that those data were obtained by using a mix of synthetic volatile fatty acids (VFAs) (75-90 % w/w) and OFMSW leachate (10-25 % w/w) during the selection procedure. Korkakaki et al.[80] reported that the use of pure leachate as the sole substrate to select PHA-accumulating bacteria was less successful than the use of a feed made mainly by synthetic VFAs, because the microbial culture selected with VFAs maintained a high PHA storage capacity even if fed with pure leachate.

PHA yield obtained, i.e. 0.44-0.52 mg mg^{-1} $COD_{cons.}$ and 0.65-1.13 mg mg^{-1} $COD_{OA\text{-}cons.}$ (Table 3.4), were very good and comparable with those reported in the literature by using other waste substrates to produce PHA (e.g. fermented olive oil mill effluent, fermented molasses, fermented cheese whey)[66,73,113]. Moreover, results from this work were higher than that reported by Zhang et al.[114] concerning the use of fermented food waste and sewage sludge as the substrate for PHA production (Table 3.4).

Table 3.4 *Comparison between studies related to PHA production using fermented OFMSW as substrate and this study*

Culture	**Substrate**	**Organic acids composition[a] (acetate/ n-butyrate/propionate/ valerate/isobutyrate/ lactate)** (%, weight basis)	**PHA content[b]** (g kg^{-1} VSS)	**Polymer composition PHB/PHA** (w/w)	**PHA Yield[c]** (mg mg^{-1} $COD_{cons.}$)	**PHA Yield[d]** (mg mg^{-1} $COD_{OA\text{-}cons.}$)	**PHA Yield[e]** (g kg^{-1} $OFMSW_{TS}$)	**Ref.**
MMC	Fermented OFMSW	30/0/70/0/0/0	600	0.77	-	-	25	[78]
MMC	Fermented food waste and sewage sludge	-	477	0.38	-	0.54	-	[114]
MMC	Fermented OFMSW	51.6/22.8/21.1/1.8/2.8/0	237±1	0.61	0.17	-	-	[79]
MMC	Leachate	-	784	-	-	-	-	[80f]
MMC	Leachate	-	778	-	-	-	-	[80g]
MMC	Percolate 1	39.8/15.3/40.3/0/0/4.6	476±41	0.57±0.04	0.44±0.17	1.13±0.18	62.9±10.5	This study
MMC	Percolate 2	37.1/10.2/46.1/2.7/2.2/1.7	465±21	0.54±0.02	0.52±0.10	0.75±0.16	114±14	This study
MMC	Percolate 3	45/20/29.4/2.8/1.1/1.7	405±28	0.57±0.01	0.50±0.10	0.65±0.06	100±6	This study
Cupriavidus necator CCGUG 52238	Fermented food waste	6/0/0/0/0/94	860	1	-	-	-	[86]
Cupriavidus necator H16	Fermented food waste	-	870	1	-	-	-	[87]

[a]See additional file 1;
[b]PHA accumulated at the end of the accumulation test referred to VSS;
[c]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{cons.}$;
[d]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{OA\text{-}cons.}$;
[e]PHA produced referred to $OFMSW_{TS}$;
[f]Accumulation test with a biomass selected with a substrate made of 90% synthetic VFAs and 10% pretreated leachate;
[g]Accumulation test with a biomass selected with a substrate made of 75% synthetic VFAs and 25% pretreated leachate.

Table 3.5 reports the mass balance of the entire process: the percolation process producing OA (First stage) plus PHA accumulation (Second stage).

Percolation trials indicated that the process parameters adopted for Trial 2 determined the highest percolate and OA production per kg of OFMSW treated, giving the best trial results among the three tested. This evidence together with the very good performance in converting OA into PHA (Second stage) using the OFMSW-supernatant$_{acc.}$ 2, resulted in the highest PHA production among the three two-stage processes, i.e. 33.22 ± 4.2 g kg^{-1} of OFMSW$_{w.w.}$, corresponding to 114.4 ± 14.5 g kg^{-1} of OFMSW$_{TS}$, almost 5 times higher than that reported by Rhu et al.[78] (25 g of PHA kg^{-1} of dry food waste).

Table 3.5 *PHA yield on OFMSW weight unit*

	FIRST STAGE		**SECOND STAGE**	**GLOBAL PROCESS**	
	Percolate produced (L kg^{-1} OFMSW$_{w.w.}$)	**OA produced**[a] (g kg^{-1} OFMSW$_{w.w.}$)	**PHA Yield**[b] (g kg^{-1} OA$_{in}$)	**PHA Yield**[c] (g kg^{-1} OFMSW$_{TS}$)	**PHA Yield**[d] (g kg^{-1} OFMSW$_{w.w.}$)
Trial 1	8.03	60.5	230±38	62.9±10.5	13.9±2.3
Trial 2	19.3	151	220±28	114±14	33.2±4.2
Trial 3	25.6	139	161±9	100±6	22.4±1.3

[a]OA expressed as acetic acid, determined according to the acid titration method[120];
[b]PHA produced referred to OA$_{in}$;
[c]PHA produced referred to OFMSW$_{TS}$;
[d]PHA produced referred to OFMSW$_{w.w.}$.

PHA characterization

Mass balance previously discussed indicated that Trial 2 gave the best results in terms of total PHA yield. Therefore, PHA characterization was focused on the products obtained in Trial 2.

Solid state ^{13}C NMR was used to evaluate the content of PHA in the generated biomass and to monitor the PHA extraction process. ^{13}C cross polarization NMR technique, based on the carbon magnetization transferred from ^{1}H protons, could not be used for quantification of different chemical species/functional groups. In contrast, single pulse excitation (SPE) NMR is based on the direct ^{13}C nuclei polarization and, even if less sensitive than CP MAS NMR, can be optimized for quantitative evaluation analysis. The most critical parameter, recycle delay, was set at 160 s, in order to cover T1 values of each ^{13}C type.[122] The obtained ^{13}C SPE NMR of the reference PHA standard, biomass enriched in PHA and the biomass after PHA extraction are shown in figure 3.3.

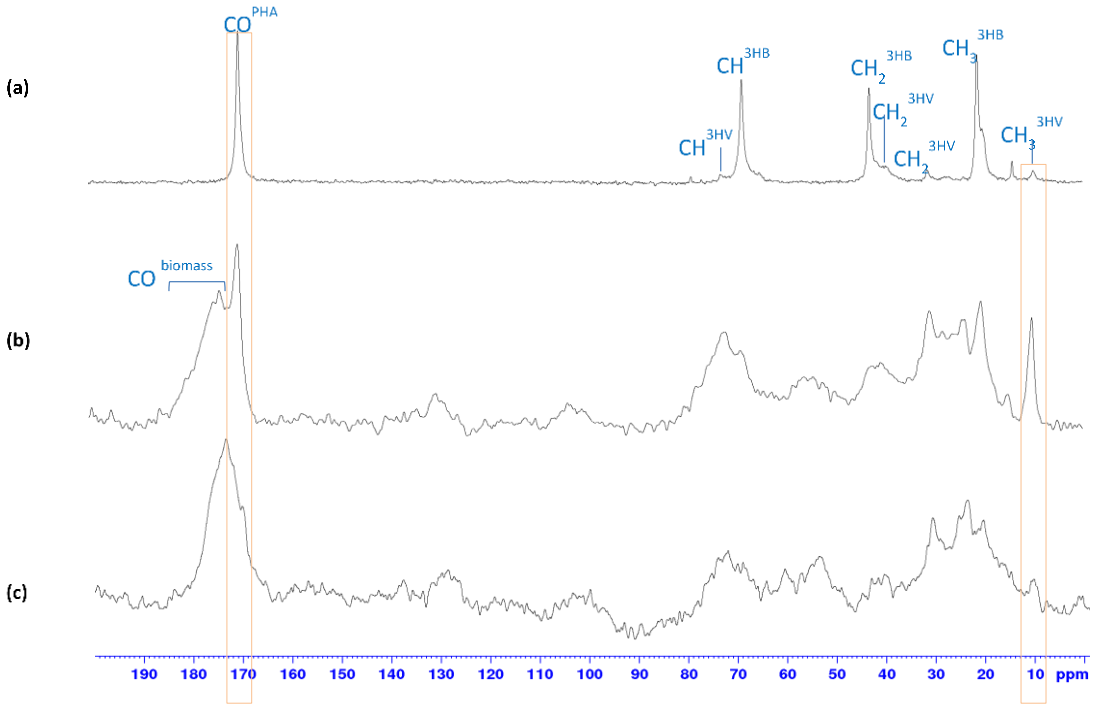

Figure 3.3 *^{13}C SPE NMR spectra of the reference pure PHA$^{88/12}$ (a), original biomass containing PHA (b) and exhausted biomass after PHA extraction (c). Frames indicate CO and $CH_3$3HV signals related to PHA polymer that can be used for monitoring the synthesis and extraction process*

Together with the PHA signals, the biomass spectrum contains signals compatible with proteins (large CO peak at 170-185 ppm), minor polysaccharides (large peak of anomeric O-C-O at 100-110 ppm), etc. It is worth noting that PHA-related peaks are sharper than the other signals, indicating that these polymers were present, at least partially, in their semi-crystalline form.

In spite of the optimized acquisition parameters, quantitative analysis of PHA in biomass was limited by strong signal overlapping, especially in the aliphatic region (0-80 ppm) (Fig. 3.3). As a tentative for PHA quantitative evaluation in the original biomass, a ratio between CO^{PHA} and $CO^{biomass}$ was estimated as 30-35 molar %, using a Topspin software for peak integral simulation. Since the result significantly depends on the estimated signal width, such an approach could not be applied for the exhausted biomass, where the CO^{PHA} peak is not sufficiently high. It still can be clearly observed from the decreased signals of CO^{PHA} (~172 ppm) (Fig. 3.3) and well isolated $CH_3{}^{3HV}$ (~10 ppm) (Fig. 3.3) that the biomass treated with chloroform contained PHA in lower quantity. Notably, even before extraction, it can be seen that the synthesized PHA are characterized by higher content of 3HV monomer that is in agreement with GC-MS data. Altogether, these data can be useful for monitoring the PHA content in biomass during their production and extraction.

In order to provide more detailed structural features of the extracted PHA polymers, solution ^{1}H NMR, GC-MS and GPC-TDA were applied along with solid state NMR. By doing so extracted PHA was characterized by ^{1}H NMR using standard PHA$^{88/12}$ for signal assignment (Fig. 3.4).

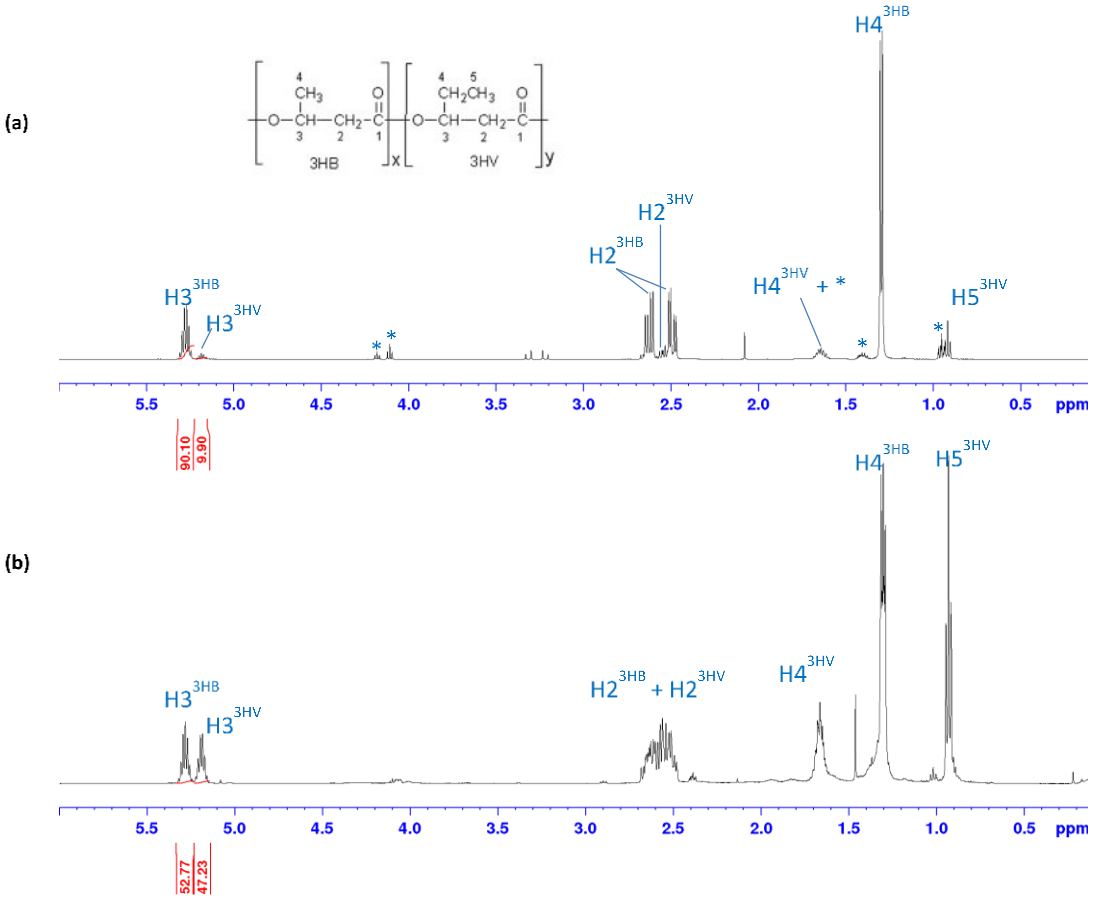

Figure 3.4 *^{1}H NMR spectra of the reference PHA$^{88/12}$ (a) and the extracted PHA (b) in $CDCl_3$. Asterisks indicate the signals related to impurities present in commercial PHA sample*

The PHA-related signals assigned using ^{1}H and COSY NMR (*not shown*) were in agreement with the previously published data.[123] The molar 3HB/3HV composition for commercial standard PHA$^{88/12}$ and biosynthesized PHA determined by ^{1}H NMR is reported in table 3.6. Notably, quantitative data obtained for the biosynthesized PHA by ^{1}H NMR (53/47 %) (Table 3.6) are also in agreement with GC-MS results.

Gel permeation chromatography with a TDA detection system was used to describe the molecular weight distribution of the biosynthesized PHA. Separation and detection conditions (including *dn/dc* calculation) were optimized using a commercial PHA$^{88/12}$ sample. Table 3.6 reports the average molecular weight Mw, polydispersity and hydrodynamic radius Rh

determined for the reference PHA$^{88/12}$ and extracted PHA sample. In spite of a similar polydispersity, the biosynthesized PHA sample was characterized by higher molecular weight and hydrodynamic radius. Mark-Houwink parameters *a* and *logK*, reflecting conformational behavior of polymers in solution, are also reported in table 3.6. The obtained *a* values for reference PHA$^{88/12}$ and extracted PHA are compatible with the values characteristic for flexible polymers in solution. Interestingly, these properties do not depend on the differences in molecular weight and chemical composition between the two samples.

Table 3.6 *Characterization of PHA produced in the accumulation stage of Trial 2 compared with commercial PHA$^{88/12}$*

Products	**HB/HV**[a] (molar ratio)	**Mw**[b] (kDa)	**Polydispersity**	**Rh**[c] (nm)	**a**[d]	***logK***[d]
Commercial reference - PHA$^{88/12}$	90/10	$2 \cdot 10^5$	1.3	14	0.70	-3.8
Extracted PHA from Trial 2	53/47	$8 \cdot 10^5$	1.4	29	0.65	-3.4

[a]Molar ratio such as detected by NMR;
[b]Molecular weight distribution determined by GPC-TDA;
[c]Hydrodynamic Radius determined by GPC-TDA;
[d]Mark-Houwink parameters *a* and *logK*.

3.5 Conclusions

Data obtained in this work can be useful as the starting point for considering both technical and economic feasibility of PHA production from OFMSW by using MMC.

The optimization of OFMSW acidic fermentation resulted a key step for the success of PHA production because it allows the production of a large amount of OA, while keeping high the performances of both food waste hydrolysis and successive fermentation to OA. Moreover, the high concentrations of OA acted to prevent biogas (methane) production from consuming the substrate to be used to produce PHA. This kind of process could be rapidly implemented in a full-scale plant by adapting dry anaerobic digestion that is commonly used to produce biogas from household wastes.

This work, for the first time, reported PHA yield referred to an OFMSW weight unit in an optimized process. Therefore, taking into consideration a PHA value of 2,600-5,800 \$ tonne^{-1},[124] data of total food waste produced in EU (87.6 million tonnes) and PHA yield obtained in this work (33.2 g kg^{-1} $OFMSW_{w.w.}$), in theory a total gross revenue of 7.6-16.9 billion \$ might be achieved.

Acknowledgements

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Supporting information

Additional file 1 *Specific acids composition of fermented OFMSW used as substrate for PHA production in literature and in this study*

Substrate	**Acetate** (%, weight basis on total OA)	**n-butyrate** (%, weight basis on total OA)	**Propionate** (%, weight basis on total OA)	**Valerate** (%, weight basis on total OA)	**Isobutyrate** (%, weight basis on total OA)	**Lactate** (%, weight basis on total OA)	**Ref.**
Fermented OFMSW	30	0	70	0	0	0	[78]
Fermented OFMSW	51.6	22.8	21.1	1.8	2.8	0	[79]
Percolate 1	39.8	15.3	40.3	0	0	4.6	This study
Percolate 2	37.1	10.2	46.1	2.7	2.2	1.7	This study
Percolate 3	45	20	29.4	2.8	1.1	1.7	This study
Fermented food waste	6	0	0	0	0	94	[86]

4. Bioconversion of giant cane for integrated production of biohydrogen, carboxylic acids and polyhydroxyalkanoates (PHAs) in a multistage biorefinery approach

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Abstract

A highly productive *Arundo donax* L. clone (Clone AD-20) was produced at full field to give 54.6 Mg total solids (TS) biomass Ha^{-1}. Biomass was chemically and enzymatically pretreated, recovering 13.9 Mg Ha^{-1} of glucose and 3.6 Mg Ha^{-1} of xylose, i.e. 3.5-4.5 more than yield typically obtained from corn stover or switchgrass. The subsequent fermentation of the liberated sugars to organic acids (OA) by dark fermentation generated yields of 3,850 Nm^3 Ha^{-1} of biohydrogen and 14.2 Mg Ha^{-1} of OAs. OAs were then used as a feed to produce polyhydroxyalkanoates (PHA), with 3-hydroxybutyrate the major monomer present (PHB > 95% PHA), from a biological process using mixed microbial culture (MMC) producing 5.04 Mg Ha^{-1} of PHA. An initial economic analysis indicated that this multi-stage biorefinery approach would result in a net revenue of 10,415 € Ha^{-1}, which is ~9-fold greater than that obtained by a traditional biorefinery producing bioethanol.

4.1 Introduction

A biorefinery is defined as the sustainable processing of biomass feedstocks into a spectrum of marketable products and energy using a multi-step processing approach.[32,125,126] Different kinds of feedstocks, such as residues from agricultural, industrial and urban sectors can be used in the biorefinery,[127,128] but in any case the biorefinery should be designed to guarantee its sustainability in terms of environment, economy and societal benefit in all of the processes involved.[128]

Certain lignocellulosic feedstocks are now becoming more attractive because of the lack of competition with the food production sector[129] and also due to the possibility of valorizing the entire aerial biomass[130]. Lignocellulosic biomasses are known to be sources of lignin, hexoses (C6) and pentoses (C5) that can be converted into large amounts of chemicals and energy carriers.[31,32]

Arundo donax L., or giant cane, is arousing interest in the scientific community for its use as a feedstock for energy sources and chemical products in a biorefinery model.[30] *A. donax* is a perennial herbaceous plant widespread in different environments and it can grow on many types of soils.[27] One of the most interesting characteristics of this plant is the amount of biomass achieved per cultivated surface area, which is much higher than that of other traditional crops. An average production of 37.7 Mg total solid (TS) Ha^{-1} was reported for a long term monitoring experiment in central Italy.[131] This data was recently exceeded by newer studies reporting yields of 60-70 Mg TS Ha^{-1} obtained in an experimental field in northern Italy.[28,29] The limited investment and the low maintenance costs necessary for *A. donax* cultivation, due to low agronomic interventions, low water and fertilizer requirements and no phytosanitary product applications,[29] makes this crop a suitable candidate for the development of an economically sustainable biorefinery.

Many studies have highlighted the possibilities of using giant cane biomass for the production of bio-energy, bio-combustibles, chemicals and other products. Biogas[28,29,33,132] and bio-ethanol[133] production, and energy from combustion processes[134] have been previously investigated for this crop. In addition, the production of chemical compounds with an economic value, such as xylo-oligosaccharides,[36] levulinic acid, γ-valerolactone,[37,135] *p*-hydroxyphenyl-propane[136] and several alkaloids[137] have all been reported for *A. donax*, as well as paper[138] and activated carbon[139] production from the raw biomass. Nevertheless, there are very few available examples of the biorefinery approach using *A. donax*. An interesting example of biorefinery from this crop is represented by the simultaneous recovery of lignin, hemicellulose, cellulose nanocrystals and silica by using sequential steps of chemical treatments and then separating the solid and liquid fractions.[38] Again, coupled with chemical and biological treatment of giant cane in order to deconstruct fibers, obtaining furfural and levulinic acids and recycling the solid residues.[34]

The cellulose and hemicellulose content in *A. donax*, coupled with the high crop yield, represent a significant potential source of glucose, xylose, arabinose and lignin.[33] Overcoming the natural recalcitrance of biomass by chemical pretreatment, it was reported the production of 11 Mg Ha^{-1} of glucose plus 4.84 Mg Ha^{-1} of xylose.[33] This large amount of sugar can then be used for different purposes, such as the production of organic molecules. Carboxylates are dissociated organic acids, which are characterized by the presence of at least one carboxyl group. Dark fermentation (DF) has been proposed as a low-cost bioprocess[140] to produce short length organic acids (OA) from wastes or biomass, i.e. the carboxylate platform,[141] together with H_2,

this latter to be valorised as co-product of OAs. The demand for polymers is continuously growing (e.g. 50 million Mg in Europe in 2016), and its negative impact due to its accumulation in the environment is well documented in the literature.[88] Recycling could limit these environmental impact of plastics, but only a small portion is currently recycled.[44] As a result, bioplastic began to be substituted for fossil-fuel-derived plastic in the market,[44] and although it represents only 1 % of the total plastic currently produced in the world, its demand is expected to grow from around 2.05 x 10^6 Mg in 2017 to approximately 2.44 x 10^6 Mg in 2022.[142] Biopolymers such as polylactic acid (PLA) and polyhydroxyalkanoate (PHA) are the main drivers of this growth.[142]

Polyhydroxyalkanoates are biodegradable polyesters of microbial origin having chemical-physical properties that are not very different from those of traditional plastics,[143] and they can replace common plastics for several applications, such as packaging films, disposable bulk materials and paper coatings. PHA production currently has high costs due to the substrate used and the use of pure microbial cultures.[62] Cost reductions can be achieved through the use of mixed microbial cultures (MMCs)[43] that convert short-length organic acids to produce PHAs.[144] This work investigates the potential for producing PHAs from organic acids via dark fermentation using sugars obtained from *A. donax* and evaluates the overall yields and productivities achieved by converting this crop in comparison with those achieved from more traditional bioenergy crops. A complete mass balance referring to the cropped surface (Ha), i.e. m^3 of H_2 Ha^{-1}, Mg of OAs Ha^{-1} and Mg of PHA Ha^{-1}, and a rough economic (revenue, € Ha^{-1}) analysis are presented as useful data to investigate the sustainability and viability of a such biorefinery.

4.2 Experimental section

Biomass production and characterization

Arundo donax L. biomass was cultivated and harvested in the A. Menozzi experimental farm (University of Milan, Landriano, PV, Italy, N 45°18'; E 9°15'). The clone used in this work (denominated AD 20 clone, UNIMI) was chosen both because of its optimal agronomic performance and its aptitude to be pretreated and subsequently saccharified.[29] The clone was harvested at the end of the growing season (October 2016), in the fourth year from transplantation (April 2012). Harvested biomass was air dried at 60 °C and then milled for further analyses.

Biomass was characterized using the standard National Renewable Energy Laboratory (NREL) procedures for compositional analysis.[145] Biomass sugar determination was performed by a

high-pressure liquid chromatography binary pump (Binary pump 1525, Waters) equipped with a 300x7.8 mm Aminex HPX-87H column and refractometer (Refractive Index 2410, Waters). A 0.004 Mol L^{-1} H_2SO_4 mobile phase was used with an operational temperature of 60 °C at a flow rate of 0.6 mL min^{-1}. Data treatment was carried out with Breeze Software (Waters, Milford, MA, USA).

Biomass pretreatment and hydrolysis

In order to convert the biomass into "ready-to-use" sugars for the subsequent steps, ionic liquid (IL) pretreatment and enzymatic saccharification processes were performed by using 1-ethyl-3-methylimidazolium acetate ($[C_2C_1im][OAc]$) at a temperature of 160 °C for 3 hours (severity factor $logR_0$ of 4.02).[33] This pretreatment and IL were chosen as gold standard as previously reported.[146]

Enzymatic hydrolysis was carried out as previously reported.[33] After hydrolysis, the biomass-buffer mixture was filtered in order to separate the insoluble fraction; the recovered liquid fraction was characterized by determining the principal chemical parameters with particular reference to the sugars content as above described. In total, 37 L of filtered hydrolyzed AD20 (AH) were produced and stocked in a freezer for the subsequent dark fermentation process.

Dark fermentation for biohydrogen production

Dark fermentations were carried out in a continuously stirred tank reactor (CSTR) with a total capacity of 4 L and a working volume of 1.5 L under continuous agitation (200 rpm). Biological processes were performed at thermophilic conditions (55 ºC) since this choice allowed a better slurry mixing, lower slurry viscosity and microbial kinetics improvement that made for faster sugar fermentation.[147] The pH range was set to 5.5-5.8 and automatically controlled and maintained by adding KOH 3 mole L^{-1}. Before biological processes were initiated, the headspace was fluxed with N_2 to ensure anaerobic conditions.

The inoculum used was a digestate coming from a full-scale anaerobic digester (55 ºC and hydraulic retention time - HRT - of 40 d) fed mainly with corn silage. Before its use, the digestate was thermally pretreated (1 hour at 100 ºC) in order to eliminate the methane-producing microorganisms and to isolate the spore-forming bacteria. The pH of the inoculum was adjusted to 5.5-5.8 with the aim of inactivating bacterial consumers of hydrogen.[148]

Four CSTR reactors were prepared and they were initially operated for 3 days in batch mode by feeding them pure glucose in order to acclimate the hydrogen-producing bacteria. Each reactor was set up with one liter of inoculum and the remaining half-liter was integrated with the feeding solution containing an organic substrate (glucose), macronutrients and

micronutrients.[149,150] After the batch-mode period, two reactors were started to be continuously fed (continuous mode) with the hydrolyzed *Arundo donax* (AH) substrate and the other two with glucose (Control). Process parameters were as follows: organic loading rate (OLR) of 13 g substrate L^{-1} d^{-1} and hydraulic retention time (HRT) of 2 days.[151,152] In addition, 50 mL d^{-1} of fresh inoculum, i.e. 10 % of the total working volume (v/v) was added to stimulate the microbial activity.[153] Processes were brought to stability, i.e. H_2 production was observed to be constant (8^{th} day), and then monitoring started for the next 25 days, for a total of 33 days.

The effluents produced daily were sampled and analyzed. Total solids (TS), pH and ammonia content (NH_3-N) were detected using standard methods[107]. Organic acid concentration and speciation were determined by using high-pressure liquid chromatography (HPLC) equipped with UV-Detector (UV detector 2487, Waters) in isocratic mode (flow rate of 0.60 ml min^{-1} and λ of 210 nm). A Rezex™ ROA-organic Acid H+ (8 %) Lc column, 300x7.8 mm, was used. Total sugar content in the media was daily determined by HPLC, as previously reported.

The gas produced was collected in a dallying Gas Sampling Bags Supel™- Inert Multi-Layer Foil and gas volume was measured with a Ritter Drum (Gas Meter TG0.5/5, Germany). The percentage of H_2 (v/v) was measured by gas chromatography (3000A-μGC, AGILENT-SRA Instruments, USA) with a thermal conductivity detector (TCD); samples were analyzed in triplicate for each reactor.

Spent liquid fractions (SLF) rich in organic acids (OAs) coming from the dark fermentations were retained, stocked and mixed.

All data were statistically analyzed by one-way ANOVA to compare means with a level of significant difference set at $p<0.05$; the Tukey test was used as the method to compare means. All statistical analyses were performed by using SPSS software (SPSS Statistics v21.0, IBM, Armonk, NY, USA).

Polyhydroxyalkanoates (PHA)

SLF coming from the dark fermentation of AH formed the substrate to produce PHA. Before its use, the SLF was submitted to a treatment consisting in a centrifugation at 20,000 g for 15 min. to reduce suspended organic carbon. The supernatant produced was used for PHA-storing bacteria selection after the C:N ratio adjustment to 10 by using NH_4Cl and its dilution with deionized water to a final COD of 1,900 mg L^{-1}.[113]

The substrate used for PHA accumulation was the same as that used for bacterial selection but, in this case, no NH_4Cl was added since it has been reported that N starvation can lead to a greater conversion of carbon into PHA because of cell growth limitation.[57] Moreover, the

substrate used for PHA accumulation was less diluted than that used for bacterial selection to avoid an excessive dilution of the biomass during the accumulation tests, with a final COD of ~7,500 mg L^{-1}.[66]

The PHA-producing bacteria selection (Mixed Microbial Culture) (MMC) was performed by using an inoculum constituted by an activated sludge (8 g total suspended solids L^{-1}) collected from the secondary sedimentation tank of a wastewater treatment plant (5.2×10^5 equivalent inhabitants) located at Peschiera Borromeo (Milan, Italy). The selection process, lasting for 50 days, was done by carrying out 10 sludge retention time cycles (SRT) of 5 d each, and it was carried out in a Sequencing Batch Reactor (SBR) (Tecnovetro, Monza, Italy) with a working volume of 1 L and by adopting an Aerobic Dynamic Feeding (ADF) strategy as previously reported.[113] The selection trend was monitored by determining the duration of the feast phase achievable by using the dissolved oxygen (DO) concentration in the selection media measured by an optical probe (FDO 925, WTW, Germany).[66]

In particular, the feast (h) to famine (h) ratio (F/F ratio) was calculated as the ratio between the lengths (h) of the two phases. For a correct selection of the PHA-storing bacteria, the F/F ratio had to be equal or less than 0.33.[45] Organic Loading Rate (OLR) was kept close to 1,900 mg COD L^{-1} d^{-1} and the C:N:P ratio was about of 100:10:4 mMole C: mMole N: mMole P. For every SRT a cycle was monitored in order to evaluate the performance of the selected culture.

The ability of the MMC to accumulate PHA was assessed by fed-batch tests carried out in a 500 mL working volume flask, with continuous aeration and stirring.[113] Six accumulation tests were performed taking selected biomass from SBR between the 7^{th} and the 10^{th} SRT. The biomass collected after PHA accumulation tests was treated and PHA was extracted as previously described.[144] The substrates fed during the selection and accumulation processes were characterized in terms of pH, TS, VS, Chemical Oxygen Demand (COD), organic acids content (acetate, butyrate, lactate, propionate and valerate), TKN, N-NH_4^+ and phosphorus (P) content.

During the selection, samples were taken during the cycle once in each SRT; every sample was characterized in terms of total suspended solids (TSS), volatile suspended solids (VSS), soluble COD, organic acids content, N-NH_4^+ content and PHA content.

During accumulation trials, samples were taken continuously in order to measure TSS, VSS, soluble COD, organic acids content and PHA content. Biomass concentration was calculated as VSS according to the standard methods.[66]

TSS and VSS were determined as previously reported.[67] Organic acids concentrations measured on filtered samples (filter diameter of 0.45 μm) were determined by high performance liquid chromatography (HPLC) as previously described.[113] PHA were determined by GC MS:[113] in this work hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through the use of two calibration curves, one for HB and another for HV using standards (1.5-15 g L^{-1}) of a commercial P(HB-HV) (88 %/ 12 %) (Sigma Aldrich, Germany), and corrected using heptadecane as the internal standard (concentration of approximately 0.1 g L^{-1}) (Sigma Aldrich, Germany).

The PHA content in cells was referred to VSS on a mass basis [PHA= (g kg^{-1} VSS)], considering VSS to be constituted by both active biomass (X) and PHA.[66] PHA was converted into COD according to the following oxidation stoichiometry: 1.67 mg COD mg^{-1} HB monomer and 1.92 mg COD mg^{-1} HV monomer.[45]

Acetate, butyrate and lactate were considered as HB precursors, valerate and propionate as HV precursors.[66] X was calculated on a COD basis considering that 1 g of X contains 1.42 g of COD.[45]

For the SBR, the specific COD consumption rate ($-q_{COD}$, mg COD mg^{-1} COD_X h^{-1}) was determined as the ratio between the amount of COD consumed during the feast phase and the time needed to deplete it per unit of active biomass. The specific PHA storage rate (q_{PHA}, mg COD_{PHA} mg^{-1} COD_X h^{-1}) was determined as the ratio between the amount of PHA stored during the feast phase and the time needed to deplete the COD per unit of active biomass. Specific growth rate (q_X, mg COD_X mg^{-1} COD_X h^{-1}) was determined as the ratio between the amount of new active biomass produced during the feast phase and the time needed to deplete the COD per unit of active biomass.[45]

For the SBR, PHA yield was calculated as the ratio between the amount of PHA stored expressed as COD and the amount of both organic acids (expressed as COD) and COD depleted, i.e. mg COD_{PHA} mg^{-1} $COD_{OA\text{-}cons.}$ and mg COD_{PHA} mg^{-1} $COD_{cons,}$. PHA yield was also calculated on organic acids fed expressed as COD (mg COD_{PHA} mg^{-1} $COD_{OA\text{-}in}$) and on COD fed (mg COD_{PHA} mg^{-1} COD_{in}). Again the growth yield was calculated as the ratio between the new biomass produced during the feast phase on a COD basis and the amount of COD depleted (mg COD_X mg^{-1} $COD_{cons.}$), as reported previously.[45]

In the accumulation batches, the specific rates and yields, except for q_X and the growth yield, that were not considered during the accumulation tests, were calculated as described before for

each pulse. In order to compare different accumulation tests, the average values for the first four pulses and for each parameter were considered.

The PHAs produced were characterized by both ^{13}C- and ^{1}H-NMR spectroscopies. In particular, Solid-state ^{13}C NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at 75.47MHz, using a 4x21 mm cylindrical zirconium rotor spun at 11,000 Hz to avoid the side bands. The ^{13}C cross polarization magic angle spinning (CPMAS) NMR spectra were acquired using recycle delay of 15s, ^{1}H 90 pulse length of 3.5 μs, 1 m contact time, acquisition time of 35 ms and from 1K to 2K scans. The 13C single pulse excitation (SPE) NMR spectra were recorded with delays of 2 s and 1K-2K scans. The chemical shifts were recorded relative to tetramethylsilane via benzene as a secondary reference. NMR experiments were performed on a Bruker 500 MHz AVANCE III NMR spectrometer (Bruker GmbH, Germany) with a 5 mm TCI cryoprobe. Deutered chloroform (99.6 %, Sigma Aldrich) was used as solvent.

^{1}H NMR spectra were recorded at 303K using recycle delay of 10 s, 64K fid size and 16 scans. 2D COSY were recorded at 303K with a recycle delay of 2 s and 8 scans. Multiple bond ^{1}H-^{13}C heteronuclear correlated experiments (HSQC) data were collected as 340 TD experiments each with 2k complex data points and 24 scans.

Molecular weights were detected by HP-SEC/TDA. The HPLC equipment consisted of a Viscotek system (Malvern Instruments Ltd, Malvern, UK) equipped with a Knauer HPLC pump K501, and a Biotech Degasi GPC degassing device. The detector system was a Viscotek mod. 302 Triple Detector Array (TDA), which is composed by Laser Light Scattering detector (90° and 7°; wavelength 670 nm), Refractive index (RI) detector (cell volume of 12 μL; light emitting diode (LED) at 660 nm wavelength) and Viscosimeter detector (four capillaries with a differential Wheatstone bridge configuration). A PL GEL 20 um MIXED A column (7.5 x 300 mm) was used. THF (Tetrahydrofuran for liquid chromatography LiChrosolv®, Sigma-Aldrich) was used as the mobile phase at a flow rate of 1 mL min^{-1}. Columns, injector and detectors were maintained at 35 °C. Samples were dissolved in chloroform at concentrations of 2-6 mg mL^{-1} and filtered on a 0.2 μm membrane before injection; injection volume was 100 μl. The system was calibrated with the PS narrow standard of known Mw, polydispersity and intrinsic viscosity (Malvern PolyCAL PS std 105k). Using extracted PHA (Arundo) at different concentrations (1.3, 2.6, 5.2 mg mL^{-1}), the differential refractive index increment (dn/dc) value was found to be equal to 0.047 and used for further calculations.

4.3 Results and Discussion

Biomass pretreatment, enzymatic saccharification and chemical characterization

Chemical characterization of *A. donax* biomass revealed a glucose, xylose, arabinose and lignin content of 36.1 ± 0.2 % TS, 19.1 ± 0.3 % TS, 1.9 ± 0.2 % TS and 24.3 ± 0.7 % TS, respectively (Table S1), in line with data reported in previous work[33]. Taking into consideration the conversion factors to be applied to convert sugars into polymers, i.e. 0.9[154], cellulose and hemicellulose contents were of 32.5 ± 0.2 % TS and 17.2 ± 0.3 % TS, respectively, in agreement with previous data[155].

IL pretreatment determined a TS loss because of the partial hemicellulose and lignin solubilization due to the pretreatment.[156] As consequence of that, pretreated biomass showed an increase of sugar concentration that was of 45.8 ± 1 % TS and of 18.7 ± 1.3 % TS for glucose and xylose, respectively, and a lignin reduction to 19.4 ± 3.1 % TS (Table S1).[33] The efficiency of the pretreatment was highlighted by both the high percentage of lignin removal after IL pretreatment, i.e. 45.6 ± 1.8 % lignin, and cellulose conversion to simple glucose after enzymatic hydrolysis, i.e. 71.2 ± 11.5 % cellulose. Hemicellulose conversion to xylose was less efficient, i.e. 32.8 ± 3.6 % hemicelluloses, due to the degradation of this polymer during pretreatment.[33] After biomass pretreatment and saccharification, the liquid fraction containing the solubilized sugars was used as feed for the subsequent dark fermentation to produce organic acids, and contained 13.9 ± 0.8 g L^{-1} of glucose and 3.5 ± 0.5 g L^{-1} of xylose.

Monitoring the dark fermentation process

The biogas produced during dark fermentation was primarily composed of H_2 and CO_2. The average H_2 content for control and AH tests was of 47.6 ± 2.5 % (v/v) and 44.6 ± 3.7 % (v/v), respectively; no CH_4 production was detected during tests in any reactors. Biogas production was stable and similar for all substrates tested, with an average H_2 production of 1.64 ± 0.26 NL L^{-1} d^{-1} and 1.57 ± 0.18 NL L^{-1} d^{-1} for the control and AH reactors, respectively (Table 4.1). The control experiment presented an H_2 molar yield of 1.78 ± 0.08 mole H_2 $mole^{-1}$ sugars. This yield was higher than that previously reported, on average, for fermented sugar-based substrates (1.48 ± 0.66 mol H_2 mol^{-1} sugars; *n=18*) (Table S2), indicating that the dark fermentation performed in this work was well designed. Hydrogen yield obtained for hydrolyzed *A. donax* was observed to be 1.7 ± 0.1 mol H_2 mol^{-1} sugars and in agreement with that obtained for the control.

Dark fermentation in this work was designed not only to produce H_2, but also OAs to be used as the substrate to produce PHAs. For the control, the main OAs produced were n-butyrate (38.3

± 2.9 mMole L^{-1}) and acetate (37.5 ± 1.0 mMole L^{-1}), followed by lactate (9.14 ± 0.7 mMole L^{-1}). Hydrolyzed *A. donax* gave similar OAs profile after DF, i.e. acetate was the main OA (37.3 ± 2.3 mMole L^{-1}) followed by n-butyrate (36.5 ± 3.1 mMole L^{-1}) and lactate (16.5 ± 1.0 mMole L^{-1}). The butyrate and acetate concentrations obtained in this study were comparable with those found previously, i.e. 30 and 40 mMole L^{-1} of butyrate and acetate, respectively.[157]

A range of OAs between 33-63 mMole L^{-1} has been reported to reduce biogas production,[158] with particular reference to butyrate[159]. Nevertheless, good results obtained in this work i.e. H_2/sugar molar ratio, seemed to exclude any toxicity effect by OAs on the fermentation processes. A restricted amount of formate was observed both for the control test (0.41 ± 0.08 mMole L^{-1}), and for the AH test (0.11 ± 0.05 mMole L^{-1}), probably due to the pathway involving H_2 consuming bacteria present.[160] Citrate (6.47 ± 0.87 mMole L^{-1}) was present only in AH due to the buffer used during biomass pretreatment. Taking into consideration metabolites produced during DF, it was possible to speculate that a mixed acid and butyric fermentation occurred.[161]

In terms of ammonia production during DF, during the stable phase both glucose and AH generated similar values, i.e. 240 ± 24 mg L^{-1} and 232 ± 45 mg L^{-1}, respectively. These data are lower than those reported to induce microbial toxicity.[162,163]

Table 4.1 *Dark Fermentation: H_2 production and chemical characterization of feeding and effluent*

		Control		Hydrolyzed *A. donax* (AH)	
		Feeding	**Effluent**	**Feeding**	**Effluent**
Daily Gas Volume	NL H_2 L^{-1} d^{-1}	-	1.92±0.21a [a]	-	1.9±0.2a
H_2	%, (v/v)	-	47.6±2.5a	-	44.6±3.7b
H_2 molar yield	mol H_2 mol^{-1} sugars	-	1.78±0.08a	-	1.70±0.06a
Feeding solution					
OLR	g sugars d^{-1}	13	0	13	0
Sugars					
Glucose	g L^{-1}	17.3±0.05	0	13.9±0.8	0
Xylose	g L^{-1}	0	0	3.5±0.54	0
Arabinose	g L^{-1}	0	0	0	0
Total sugars	g L^{-1}	17.3±0.05a	0	17.4±0.12a	0
Organic acids					
Citrate		0	0	7.85.±0.9	6.47±0.87
Formate		0	0.41±0.08	0	0.11±0.05
Acetate		0	37.5±1	0	37.3±2.30
Propionate		0	0	0	0
Isobutyrate	mmol L^{-1}	0	0.12±0.07	0	0
n-butyrate		0	38.3±2.9	0	36.5±3.1
Isovalerate		0	0	0	0
Lactate		0	9.14±0.7	0	16.5±1
Total OA		0	85.5±15.8	0	96.9±10.2
pH	-	6.25±0.15a	5.76±0.6a	4.37±0.11b	5.78±0.11a
NH_3	mg L^{-1}	216±12a	240±24a	158±15b	232±45a
TKN	g L^{-1}	0.24±0.02a	0.55±0.11a	0.30±0a	0.68±0.08a
C:N	-	29±0a	12±0a	20±0b	13±1.5a

[a]Averages followed by the same letter are not statistically different for a $p<0.05$.

Total OAs yield obtained from DF of the hydrolyzed *A. donax* was significant, i.e. 0.64 g OA g^{-1} COD (i.e. 0.81 g OA g^{-1} VS) (Table 4.2), and much higher than OAs yields typically obtained by DF that are reported in the literature (Table 4.2). The only exception was for OA generated by DF of pure sugar (xylose) using pure microbial culture and a long retention time (22 d).[164]

Table 4.2 *Organic acids yield obtained in this work by dark fermentation in comparison with data from the literature*

Feedstock	**Culture**	**HRT** (d)	**Operation modality**	**OLR**	**Temperature** (°C)	**Total OA**[a] ($g\ L^{-1}$)	**Yield** ($g\ OA\ g^{-1}\ COD$)	**Ref.**
FW[b]	mixed	3-3.3	continuous	16.8-17 kg VS $m^{-3}\ d^{-1}$	55	12.3-13.7	0.22-0.23	[165]
WAS[c]	mixed	4.6-5.9	continuous	1.2-1.9 kg VS $m^{-3}\ d^{-1}$	35	3.2-7.5	0.21-0.33	[165]
FW	mixed	2	continuous	15 kg COD $m^{-3}\ d^{-1}$	28	6.3	0.42	[166]
AH[d]	**mixed**	**2**	**continuous**	**13 g d^{-1}**	**55**	**7**	**0.64**	**This study**
							Yield ($g\ OA\ g^{-1}\ VS$)	
WAS+FW	mixed	4	continuous	14.4 $g\ L^{-1}$	20	8.33	0.57	[167]
FW	mixed	8	continuous	13 $g\ L^{-1}$	35	28.9–30	0.29-0.3	[168]
CS[e]/PM[f]	mixed	6	continuous	2-5 % TS	37	31.8	0.55	[169]
KW[g]	mixed	4	batch	48.2 g VS L^{-1}	37	9.81	0.2	[170]
WAS + HPB[h]	mixed	21	continuous	19.4 $g\ L^{-1}$ SV	35	5.3	0.27	[171]
FW	mixed	5	continuous	11 g TS $L^{-1}\ d^{-1}$	35	21.4	0.41	[172]
Xylose	pure	22	continuous	50 $g\ L^{-1}$	36	59	0.99	[164]
AH	**mixed**	**2**	**continuous**	**13 g d^{-1}**	**55**	**7**	**0.81**	**This study**

[a]OA: organic acids;
[b]FW: food waste;
[c]WAS: waste activated sludge;
[d]AH: arundo hydrolized;
[e]CS: corn stover;
[f]PM: pig manures;
[g]KW: kitchen wastes;
[h]HPB: henna plant biomass.

PHA production from dark fermentation effluent

PHA-storing bacteria selection performed on the spent liquid fraction coming from the dark fermentation of hydrolyzed Arundo biomass showed good performance, i.e. the average feast to famine ratio (F/F) was equal to 0.06 with a standard deviation of 0.03 (Figure 4.1). This result was much lower than 0.33, this value being assumed as the limit up to which a good selection is obtainable.[45] Microbial growth yield referred to COD consumed was equal to 0.38 ± 0.03 mg $COD_X\ mg^{-1}\ COD_{cons.}$, clearly lower than that reported for PHA storage i.e. 0.54 ± 0.11 mg $COD_{PHA}\ mg^{-1}\ COD_{cons.}$, indicating a preferred utilization of the carbon consumed for polymer storage rather than for microbial growth during the feast phase.

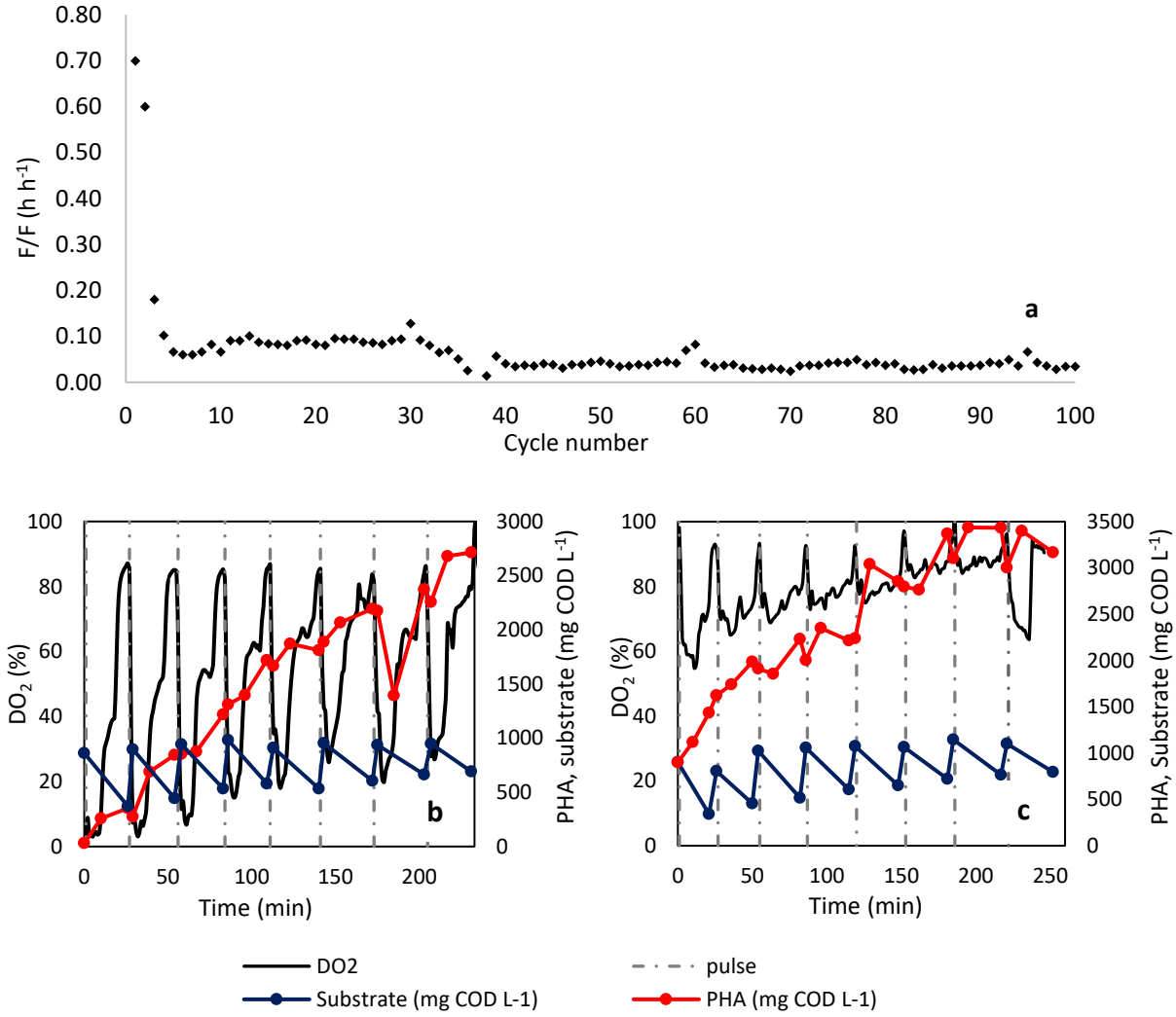

Figure 4.1 *Feast to famine ratio (F/F) during all the duration of the selection process (a); DO_2, substrate and PHA trend during two accumulation tests performed between the 7th SRT (b) and 10th SRT (c)*

The PHA stored in the microbial cells at the end of the accumulation tests (Figure 4.1) was, on average, equal to 543 ± 106 g PHA kg^{-1} VSS, with PHA being primarily composed of HB (96 %, w/w).

PHA content observed in this work was in agreement with the average data reported in the literature by other authors using fermented vegetable substrates (581 ± 193 g PHA kg^{-1} VSS) (Table 4.3). The higher PHA content reported in table 4.3 are attributed to both longer selection processes performed (5 to 8 months vs. 50 days adopted in this work) and the higher temperature used (30 °C vs. 23 °C). Taking into consideration that about 77 % of the COD consumed ($COD_{cons.}$) consisted of organic acids (COD_{OAcons}), it can be deduced that PHA was mainly produced starting from OAs, of which the yield, referred to COD_{OA}, resulted of 1.15 ±

0.21 mg COD_{PHA} mg^{-1} $COD_{OA\text{-}cons.}$, that is 0.85 ± 0.21 mg COD_{PHA} mg^{-1} $COD_{cons.}$. Both values reported were much higher than those cited in the literature for similar substrates (Table 4.3). These data confirm that OAs were the preferred carbon sources for PHA production by mixed microbial cultures (MMC).[81,82] Again, the good PHA yield obtained in this work, i.e. 290 g PHA kg^{-1} of sugars, can be ascribed to the use of OAs as feed, as OAs have been reported to be direct precursors of PHA monomers.[43,53,65] The use of sugars instead of OAs as feed generally leads to a microbial population (MMC) enriched in other bacteria than just the PHAs-storing ones (i.e. glycogen-storing bacteria), reducing final PHA content.[53,108]

Considering the total substrate fed to the system, PHA yield was 0.65 ± 0.11 mg COD_{PHA} mg^{-1} $COD_{OA\text{-}in}$ and 0.41 ± 0.13 mg COD_{PHA} mg^{-1} COD_{in}. These results indicate that about half of the COD was not used to produce PHA. This means that the OAs present in the feed (71 % of the total COD fed) were not completely consumed because of the reduced culture performance observed during the production runs. In fact, when the bacteria reached values close to the maximum theoretical polymer content, they started to reduce their ability to consume OAs. In any case, PHA storage yield on COD fed obtained in this study was, on average, 241 ± 75 g PHA per kg of COD, which was in agreement with results reported by using fermented paper mill wastewater (containing lignocelluloses) as substrate[82].

Taking into consideration PHA storage yield in relation to COD fed, and dark fermentation yield in terms of COD produced in relation to sugars fed, a total PHA production of 290 g per kg of sugars was reached, i.e. 90 g of PHA kg TS^{-1} *A. donax*. This yield was in agreement with values previously reported by us, i.e. 92 g PHA kg^{-1} TS[144] and 65 g PHA kg^{-1} TS[113], by using the organic fraction of municipal solid waste (OFMSW) and cheese whey, respectively. These results seem to indicate that it is not the complexity of the raw biomass used that affect final PHA yield, but the efficacy of the processes that preceded PHA production, i.e. biomass pretreatment (deconstruction), hydrolysis (enzymatic), and fermentation (dark fermentation).

Table 4.3 *Comparison among parameters characterizing MMC selection (SBR) and PHA accumulation processes performed in this study and in other studies carried out with fermented vegetable effluents*

	Substrate	PHA content (g PHA kg^{-1} VSS)	Polymer composition (ΔHB/ΔHV) (%, w/w)	PHA characterization	$-q_{COD}$[a] (mg COD mg^{-1} COD_X h^{-1})	q_{PHA}[b] (mg COD_{PHA} mg^{-1} COD_X h^{-1})	q_X[c] (mg COD_X mg^{-1} COD_X h^{-1})	PHA Yield[d] (mg COD_{PHA} mg^{-1} $COD_{OA\text{-}cons.}$)	PHA Yield[e] (mg COD_{PHA} mg^{-1} $COD_{OA\text{-}in}$)	PHA Yield[f] (mg COD_{PHA} mg^{-1} $COD_{cons.}$)	PHA Yield[g] (mg COD_{PHA} mg^{-1} COD_{in})	PHA Yield[h] (g PHA kg^{-1} OA_{in})	PHA Yield[i] (g PHA kg^{-1} COD_{in})	PHA Productivity (g PHA L^{-1} d^{-1})	Growth Yield[j] (mg COD_X mg^{-1} $COD_{cons.}$)	Ref.
SBR	PFA[k]	132.3±23.2[l]	95.3±0.6/ 4.7±0.6	-	294.7±15.6 (0.25±0.01 mmol C $mmol^{-1}$ C_X h^{-1})	159.6±26.4 (0.14±0.02 mmol C_{PHA} $mmol^{-1}$ C_X h^{-1})	112.1±3.2	0.87±0.31	0.35±0.14	0.54±0.11	0.26±0.08	-	-	-	0.38±0.03 (0.45±0.04 mmol C_X $mmol^{-1}$ $C_{cons.}$)	This study
Accumulation	PFA	543±106[m]	96±1/4±1	$1*10^6$ kDa (MW)[n] 2.1 (Polidispersity) 30 nm (Rh)[o] 0.8 (a)[p] -4.5 (log K)[q]	413.3±105.5 (0.38±0.07 mmol C $mmol^{-1}$ C_X h^{-1})	346.6±110.1 (0.31±0.09 mmol C_{PHA} $mmol^{-1}$ C_X h^{-1})	-	1.15±0.21 (1.21±0.22 mmol C_{PHA} $mmol^{-1}$ $C_{OA\text{-}cons.}$, 0.97±0.18 g PHA g^{-1} OA-cons.)	0.65±0.11	0.85±0.21 (0.9±0.2 mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	0.41±0.13	450±89	241±75	14.2±4.3	-	This study
	FPOME[r]	640[m]	77/23	-	-	-	-	0.59 (g PHA g^{-1} OA-cons.)	-	-	-	-	-	-	-	[76]
	FPMWW[s]	768[m]	86/14	-	-	-	-	-	-	0.8	-	-	-	2	-	[81]
	FWME[t]	270[m]	81/19	-	0.151 (mmol C $mmol^{-1}$ C_X h^{-1})	0.04 (mmol C_{PHA} $mmol^{-1}$ C_X h^{-1})	-	-	-	0.35 (mmol C_{PHA} $mmol^{-1}$ $C_{cons.}$)	-	-	-	-	-	[83]
	FPMWW	688[m] (482 g PHA kg^{-1} TSS)	39/61	-	0.09±0.01 (mmol C_{OA} $mmol^{-1}$ C_X h^{-1})	0.06±0.01 (mmol C_{PHA} $mmol^{-1}$ C_X h^{-1})	-	0.66 (mmol C_{PHA} $mmol^{-1}$ $C_{OA\text{-}cons.}$)	-	-	-	-	270	-	-	[82]
	FOOME[u]	540[m]	89/11	-	-	417±42	-	1	-	-	-	-	-	-	0.18 (mmol C_X $mmol^{-1}$ $C_{OA\text{-}cons.}$)	[73]

[a]Specific COD consumption rate;
[b]Specific PHA storage rate;
[c]Specific growth rate during feast phase;

[d]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{OA\text{-}cons.}$;
[e]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{OA\text{-}in}$;
[f]PHA storage yield expressed as COD (COD_{PHA}) referred to $COD_{cons.}$;
[g]PHA storage yield expressed as COD (COD_{PHA}) referred to COD_{in};
[h]PHA produced referred to OA_{in};
[i]PHA produced referred to COD_{in};
[j]Growth yield during feast phase expressed as COD (COD_X) referred to $COD_{cons.}$;
[k]PFA: pretreated fermented Arundo;
[l]PHA stored at the end of the feast phase referred to VSS;
[m]PHA accumulated at the end of the accumulation test referred to VSS;
[n]MW: molecular weight;
[o]Rh: hydrodynamic radius;
[p]a: Mark-Houwink parameter;
[q]logK: Mark-Houwink parameter;
[r]FPOME: fermented palm oil mill effluent;
[s]FPMWW: fermented paper mill wastewater;
[t]FWME: fermented wood mill effluent;
[u]FOOME: fermented olive oil mill effluent.

Solid state ^{13}C NMR was used to characterize the extracted PHA polymers. ^{13}C cross-polarization NMR technique, based on the carbon magnetization transferred from ^{1}H protons, was optimized for the identification of major signals related to PHA polymers. The obtained ^{13}C CP-MAS NMR of reference $PHA^{88/12}$ standard (3-hydroxyvalerate -3HV- content 12 % mole) was compared to extracted PHA (Arundo) as reported in figure 4.2 (top-left). Notably, PHA extracted from Arundo was mainly composed by the 3-hydroxybutyrate monomer (3HB). In order to provide more detailed structural features of the extracted PHA polymers solution, ^{1}H NMR, was applied along with solid state ^{13}C NMR.

Reference $PHA^{88/12}$ was used for signal assignment in the ^{1}H NMR spectra. 3-Hydroxybutyrate and 3-Hydroxyvalerate related signals were assigned by ^{1}H and COSY NMR (Figure 4.2, top-right). The results obtained were in agreement with the previously published data.[123] ^{1}H, COSY NMR and 2D HSQC NMR (Figure 4.2, top-right and bottom-left) recorded on PHA extracted from Arundo confirmed that 3-hydroxybutyrate (3HB) represented the mayor monomer in the structure (>95 %), while the amount of 3-hydroxyvalerate (3HV) was lower than 5 %. Quantitative estimation of PHA monomers was performed by ^{1}H NMR analysis using the ratio of signals related to H3 of 3HB and 3HV at 5.25 and 5.16 ppm, respectively. The same calculation was made for reference $PHA^{88/12}$ and biosynthesized PHA from different sources. From the comparison of the ^{1}H NMR spectra (Figure 4.2, bottom-right) it can be seen that the different composition of the polymers was due mainly to the different organic acids contained in the substrate fed to the PHA storing bacteria. In particular, the carbon sources used in this study and in a previous work (fermented cheese whey)[113] contained 100 % of HB precursors, which led to primarily HB found in the polymer stored. In contrast, by using a substrate composed of 50 % of HB precursors (percolate of OFMSW), a previous study reported only 50 % of HB in the polymer stored.[144]

The molecular weight distribution of PHA samples was analyzed by HP-SEC-TDA. Separation and detection conditions, including *dn/dc* value, were optimized and tested using an extracted PHA (Arundo) sample. Average molecular weight (Mw), polydispersity (Mw/Mn) and hydrodynamic radius (Rh) determined for the commercial reference $PHA^{88/12}$ and PHA extracted from *A. donax* was found to be $2x10^5$ Da, 1.3 and 12 nm, and $1x10^6$ Da, 2.1 Mw/Mn and 30 nm, respectively. The biosynthesized PHA was characterized by higher molecular weight and hydrodynamic radius than the commercial reference sample. Mark-Houwink parameters *a* and *LogK*, reflecting conformational behaviour of polymers in solution were detected as well. The *a* values obtained for reference $PHA^{88/12}$ (*a*= 0.80; *logK*= - 4.5) and

extracted PHA from *A. donax* (*a*= 0.70; *logK*= - 3.9) are consistent with the values attributed to flexible polymers in solution. Interestingly, these properties did not depend on the differences in molecular weight between the two samples analysed.

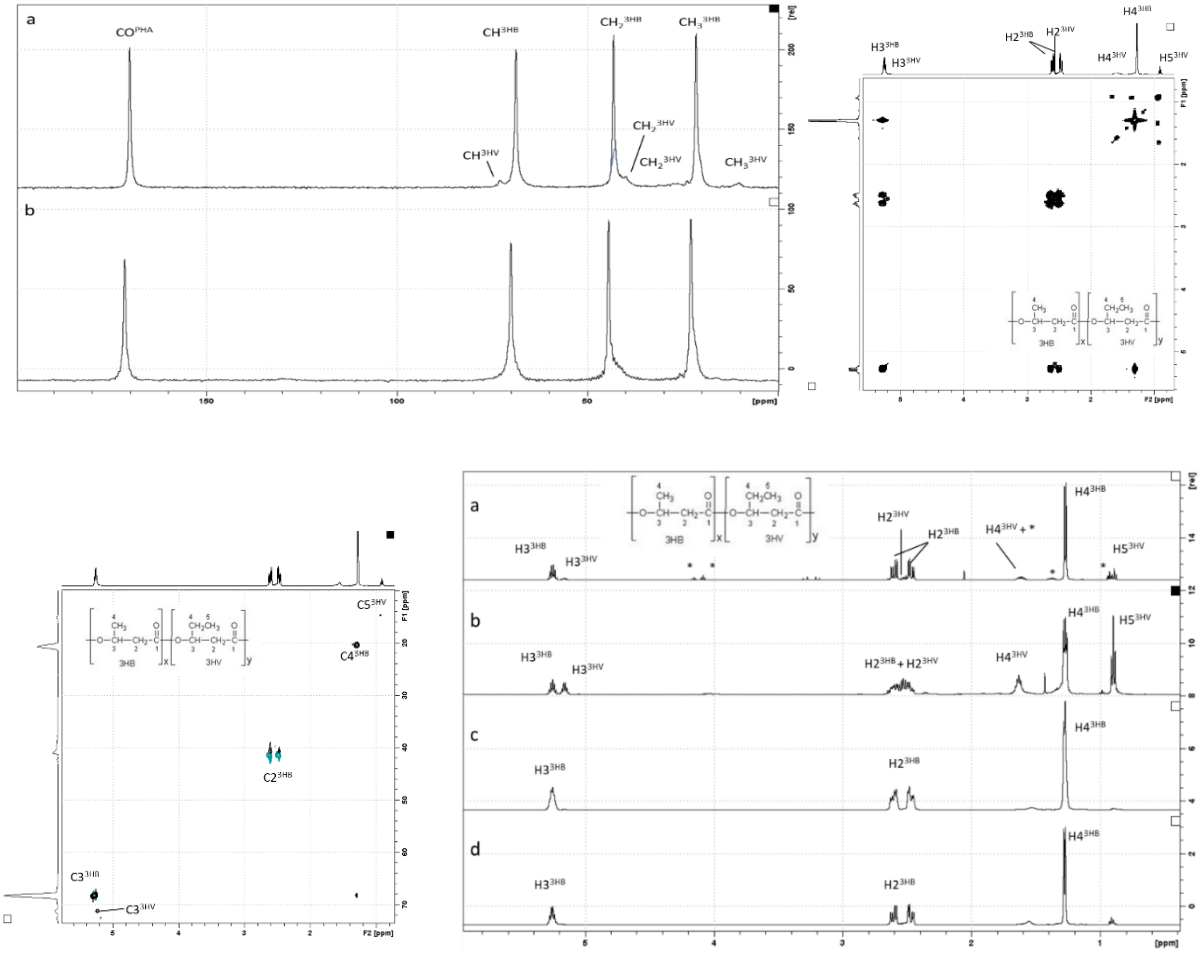

Figure 4.2 *CP-MAS ^{13}C NMR spectra of reference PHA$^{88/12}$ (a) and fermented-Arundo-PHA (b) (top-left); COSY NMR spectra of the fermented-Arundo-PHA in $CDCl_3$ (top-right); HSQC NMR spectra of the fermented-Arundo-PHA in $CDCl_3$ (bottom-left); ^1H NMR spectra of the reference PHA$^{88/12}$ (a), PHA obtained from OFMSW[140] (b), and PHA obtained from fermented cheese whey[111] (c) and the fermented-Arundo-PHA (d) in $CDCl_3$ (bottom-right). Asterisks indicate signals related to impurities present in reference PHA sample*

4.4 Mass balance and economic evaluations

The *Arundo donax* L. clone produced 54.6 Mg Ha^{-1} of dry biomass, and these high levels of productivity are well documented in the literature.[29,33] This biomass led, after pretreatment and hydrolysis processes, to the production of 13.9 Mg Ha^{-1} of glucose and 3.6 Mg Ha^{-1} of xylose (Figure 4.3). These values are comparatively significant as *A. donax* was able to produce about 3-5 times more sugar Ha^{-1} than those obtained, for example, from switchgrass and corn stover,[29] and confirm the potential of this crop to enable the realization of a sustainable and viable

lignocellulosic biorefinery. The subsequent use of the sugars mixture to sustain the dark fermentation process gave an amount of bio-H_2 that was quantifiable as 3,850 Nm^3 H_2 Ha^{-1} and 14.2 Mg Ha^{-1} of OAs (Figure 4.3). The OAs recovered after DF were then used as feed to produce PHAs by using MMC. Taking into consideration both crop yield and subsequent transformation of biomass into PHA (Table 4.3), a potential production of 5.04 Mg Ha^{-1} of PHA (Figure 4.3) was calculated.

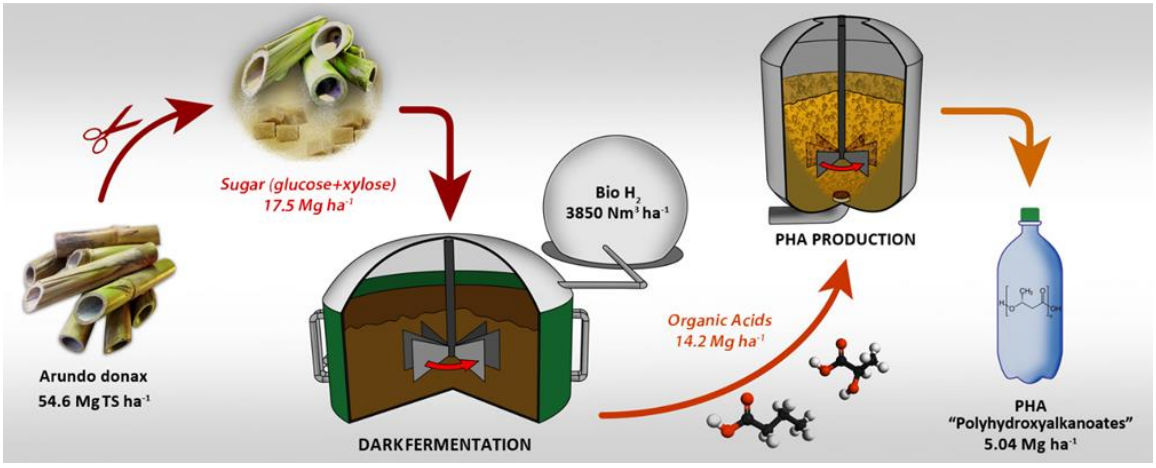

Figure 4.3 *Arundo donax biorefinery concept and mass balance*

Now taking into consideration potential productivity of *A. donax* in terms of H_2 and PHA per surface area obtained in this work, the costs both to produce biomass and to convert it into final products, and the products' selling price (Table 4.4), a brief economic analysis was made of the proposed biorefinery. The results from this economic analysis were compared with alternative and documented uses of *A. donax*, i.e. biogas and bioethanol production, representing simple biorefinery approaches (Table 4.4). Results were interesting: bioethanol appeared to be sustainable with a total revenue of 73.4 € Mg^{-1} TS biomass, i.e. net revenue of 19.5 € Mg^{-1} TS biomass (calculated from Table 4.4). The large amount of biomass produced per Ha generated a total revenue per surface area of 4,012 € Ha^{-1}, (net revenue of 1,068 € Ha^{-1}), significantly higher than those obtained from corn stover, i.e. 1,032 € Ha^{-1} (total biomass revenue of 120-130 € Mg^{-1} TS biomass[173] and corn stover production of 8.6 Mg Ha^{-1} [33]). Biomethane production from *A. donax* gave a negative economic balance, although the total energy produced per Ha^{-1} was higher than that of bioethanol. The low methane price was responsible for that and confirmed that biogas/biomethane production needs to be supported by government benefits or other income such as for example a waste tariff.

Increasing the number of products derived from lignocellulosic biomass by a partial or complete biorefinery process has been reported to increase total and net revenue.[173]

In this work, comparing a simple biorefinery (bioethanol) (Table 4.4) with the partial multi-stage biorefinery proposed (H2 plus PHA), total revenue increased by 4.9-fold and the net revenue by 9.7-fold.

The sustainability of the *A. donax* biorefinery was more evident when this feedstock was compared with the most common feedstocks proposed to develop lignocellulose biorefinery, i.e. corn stover and switchgrass (Table 4.4).[174,175,176] The high biomass yield reported for *A. donax* assured a total PHA production 3.87 and 3.31 higher than that estimated for corn stover and switchgrass, respectively (Table 4.4). This fact allowed getting a net revenue that increased with respect to those calculated for these two feedstocks by 3.38 and 3.02-fold (Table 4.4). These results indicated that the choice of crop becomes important for the development of a sustainable biorefinery and that *A. donax* is an excellent candidate to get this thanks to both high biomass (and products) production performance, both low crop cost and environmental impact.[177]

Table 4.4 *Comparison of hydrogen and PHA production with biomethane and ethanol in terms of product yield, energy recovered, production costs and estimated net revenue*

	Product yield	Energy recovered	Unitary production cost	Total cost per Ha	Unitary selling price	Total revenue	Net revenue
A. donax							
Biomethane	9,518[a] $Nm^3 CH_4$ Ha^{-1}	301[a] GJ Ha^{-1}	-0.57[a] € Nm^{-3} CH_4	5,425 € Ha^{-1}	0.19[b] € Nm^{-3}	1,808 € Ha^{-1}	-3,617 € Ha^{-1}
Ethanol	11,324[c] L Ha^{-1}	239[d] GJ Ha^{-1}	-0.26[e] € L^{-1}	2,944 € Ha^{-1}	0.35[f] € L^{-1}	4,012 € Ha^{-1}	1,068 € Ha^{-1}
			This work				
A. donax							
Crop	54.6 Mg TS Ha^{-1}			1,000[g] € Ha^{-1}			
Hydrogen	3,850 $Nm^3 H_2$ Ha^{-1}	42.4[h] GJ Ha^{-1}	-0.82[i] € Nm^{-3} H_2	3,157 € Ha^{-1}	0.63[j] € Nm^{-3}	2,426 € Ha^{-1}	-731 € Ha^{-1}
PHA	5.04 Mg Ha^{-1}	111[k] GJ Ha^{-1}	-1,000[l] € Mg^{-1}	5,040 € Ha^{-1}	3,410[m] € Mg^{-1}	17,186 € Ha^{-1}	12,146 € Ha^{-1}
Whole biorefinery (H_2 + PHA)		153 GJ Ha^{-1}		-9,197 € Ha^{-1}		19,612 € Ha^{-1}	10,415 € Ha^{-1}
Corn stover							
Crop	8.6[n] Mg TS Ha^{-1}			535[o] € Ha^{-1}			
Hydrogen	1,036 $Nm^3 H_2$ Ha^{-1}	11.4[h] GJ Ha^{-1}	-0.82[i] € Nm^{-3} H_2	850 € Ha^{-1}	0.63[j] € Nm^{-3}	653 € Ha^{-1}	-197 € Ha^{-1}
PHA	1.3 Mg Ha^{-1}	30[k] GJ Ha^{-1}	-1,000[l] € Mg^{-1}	1,360 € Ha^{-1}	3,410[m] € Mg^{-1}	4,638 € Ha^{-1}	3,278 € Ha^{-1}
Whole biorefinery (H_2 + PHA)		41.4 GJ Ha^{-1}		-2,745 € Ha^{-1}		5,291 € Ha^{-1}	3,081 € Ha^{-1}
Switchgrass							
Crop	10[p] Mg TS Ha^{-1}			433[q] € Ha^{-1}			
Hydrogen	1,164 $Nm^3 H_2$ Ha^{-1}	12.8[h] GJ Ha^{-1}	-0.82[i] € Nm^{-3} H_2	954 € Ha^{-1}	0.63[j] € Nm^{-3}	733 € Ha^{-1}	-221 € Ha^{-1}
PHA	1.52 Mg Ha^{-1}	33.4[k] GJ Ha^{-1}	-1,000[l] € Mg^{-1}	1,524 € Ha^{-1}	3,410[m] € Mg^{-1}	5,183 € Ha^{-1}	3,659 € Ha^{-1}
Whole biorefinery (H_2 + PHA)		46.2 GJ Ha^{-1}		-2,911 € Ha^{-1}		5,916 € Ha^{-1}	3,438 € Ha^{-1}

[a]Biomethane production yield, total energy produced and biomethane production cost[28];
[b]Biomethane selling price[178];
[c]Ethanol production yield calculated considering sugars conversion as 0.51 g Ethanol g^{-1} sugar[179];
[d]Total energy produced with ethanol calculated by considering a LHV of 26.84 MJ kg^{-1};
[e]Ethanol production cost[180];
[f]Ethanol selling price[173], in agreement with ethanol price commodity on 5/10/2018;
[g]Biomass production costs[28];
[h]Total energy produced by H_2 calculated by considering LHV of 11 MJ Nm^{-3} [180];
[i]Hydrogen production cost composed by cost for pre-treatment and cost in performing the dark-fermentation[181,182];
[j]Hydrogen selling price[183];
[k]Total energy produced calculated by considering a PHA-LHV of 22 MJ kg^{-1} [184];
[l]PHA/PHB production cost, referred just to polymer production without considering substrate cost (already considered), recalculated from CalRecycle[185];
[m]PHA/PHB selling price reported by CalRecycle[185];
[n]Biomass yield of corn-stover as previously reported[185];
[o]Corn-stover production cost as reported by Thompson and Tyner[174];
[p]Biomass yield of switchgrass as reported by Shi et al.[175];
[q]Switch-grass production cost as reported by Khanna et al.[176].

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Supporting information

Table S1 *Compositional analysis and mass balance of Arundo donax L. pretreatment and hydrolysis*

	Biomass loss	Glucose	Xylose	Arabinose	Lignin
			(% TS)		
[a] **Untreated**		36.1±2	19.1±0.3	1.9±0.2	24.3±0.7
After [C_2C_1im][OAc]		45.1±3.2	18.7±1.3	0	19.4±3.1
Enzymatic hydrolysis		43.1±2.3	15.9±1.2	0	-
After hydrolysis		35.8±2.7	4.1±0.9	0	31.1±0.2
			(g 100 g^{-1} TS)		
[b] **Untreated**		36.1±2	19.1±0.3	1.9±0.2	24.3±0.7
After [C_2C_1im][OAc]	31.7±3.9	30.8±3.3	12.8±1.4	0	13.2±2.4
Enzymatic hydrolysis	-	25.7±2.8	6.26±0.7	0	-
After hydrolysis	-	14.8±2.1	2.5±0.6	0	13.2±0.6

[a]Compositional analysis of untreated and pretreated biomass as a function of process step;
[b]Mass balances of sugars and lignin for untreated and pretreated biomass (data referred to 100 g of TS of untreated biomass).

Table S2 *Literature data for biohydrogen production and biohydrogen yield obtained adopting different operating conditions. Average H_2 yield of 1.48±0.66 (n=18)*

Reactor Type	T (°C)	HRT (h)	OLR (g sugars L^{-1})	H_2 production (L L^{-1} day^{-1})	H_2 yield (mol H_2 mol^{-1} sugars)	Ref.
CSTR	37	12-0.5	-	76.8	1.93	[186]
Fixed Bed Reactor	25	2	2	-	1.51	[160]
Fluidized Bed Reactor	30	6	5	1.92	0.44	[187]
CSTR	37	10	55.4	8.82	2.4	[188]
CSTR	37	6	139	25.1	2.88	[188]
CSTR	37	2	10	46.6	1.39	[189]
CSTR	37	0.5	10	76.8	1.81	[189]
Fixed Bed Reactor	30	4	30	-	1.33	[190]
Fixed Bed Reactor	30	2	60	-	1.1	[190]
Fixed Bed Reactor	30	1	120	-	1.06	[190]
CSTR	35	24	30	2.9	0.61	[191]
Upflow Reactor	55	2	34	9.33	1.76	[192]
Chemostat Reactor	35	12	20	2.24	0.7	[193]
Chemostat Reactor	50	12	20	5.6	1.4	[193]
CSTR	35	13.3	20	-	2.35	[194]
CSTR	35	2	20	-	1.48	[194]
CSTR	35	2-12	10	3.02-4.53	1.7	[195]
UASB	35	2-12	10	2.79-10.2	0.7	[195]

5. Bio-electrorecycling of carbon dioxide into bioplastics

Pepè Sciarria, T., Batlle-Vilanova, P., Colombo, B., Scaglia, B., Balaguer, M.D., Colprim, J., Puig, S., Adani, F. (2018). Bio-electrorecycling of carbon dioxide into bioplastics. *Green Chemistry*, vol. 20, p. 4058-4066.

Abstract

The rise of carbon dioxide (CO_2) emissions and the accumulation of non-biodegradable plastics in the environment are leading to an environmental crisis. Thus, the bio-electro recycling of recalcitrant CO_2 as feedstock to produce bioplastics could be an interesting solution to explore. In this work, a bioelectrochemical reactor was used to carry out microbial electrosynthesis (MES) of volatile fatty acids (VFAs) from CO_2 and then, those VFAs were used to produce polyhydroxybutyrate (PHB) by using a pre-selected mixed microbial culture (MMC). During MES (cathode potential at −0.8 V vs SHE), CO_2 fixation efficiency, i.e. carbon (C) transferred to final products was of 73 % C_{CO2}, with a final values of 43.7 and 103 mmol of C produced for acetate and butyrate. The VFAs obtained were extracted and concentrated by liquid membrane extraction getting a broth with a C concentration of approximately 400 mmol C L^{-1} (~65% butyrate), to be used as feeding for PHA producing bacteria. During the PHA accumulation a maximum of 74.4 ± 6 g PHA 100 g^{-1} VSS was obtained with a PHA yield (Y_{tot}) of 0.77 ± 0.18 mmol C_{PHA} $mmol^{-1}$ C_{fed}. The process efficiency calculated taking into account the PHA yield on C inlet as CO_2 was of 0.50 ± 0.07 mmol C_{PHA} $mmole^{-1}$ C_{CO2}. In terms of C conversion, 0.41 kg of carbon as PHA were obtained per 1 kg of carbon as CO_2 inlet to the entire system. These results stablish a sustainable way to convert a greenhouse gas as CO_2 into environmental friendly bioplastics.

5.1 Introduction

Nowadays the world is facing an environmental crisis because of the increase of CO_2 and other greenhouse gas (GHG) emissions and the accumulation in the environment of non-biodegradable, fossil-fuel plastics. CO_2 rise, due primarily to fossil fuel combustion (77 % of the total emission), contributes to the global warming, that could lead to dramatic effects on earth's climate within the next decades.[196,197,198]

The use of CO_2 as feedstock for chemicals and bio-based products is gaining attention as demonstrated recently by the Bio-Based Industries Joint Undertaking (BBI JU), which aims to

moving towards a bio-based economy in Europe using domestic renewable raw materials to locally produce food, feed, chemicals, materials and fuels.[199,200] Moreover, the use of CO_2 as feedstock is twice advantageous because it is greatly available and does not compete with food supply chain.[201]

Biological CO_2 sequestration methods using microorganisms as catalysts could lead to important advances compared to conventional CO_2 capture methods, which usually are considered expensive.[202] In this sense, microbial electrosynthesis (MES) is based on the use of bioelectrochemical systems (BES), where the CO_2 is reduced into multi carbon organic compounds by microorganisms, which are employed as biocatalysts in either the cathode or anode to achieve electricity-driven synthesis of a wide variety of compounds.[203]

Plastic production (mainly from fossil fuels) reached 335 million tons on 2016 worldwide and it expected to continue on a positive trend (+1.5 %) in the following years.[44] Part of these plastics are not produced to become waste, but to provide service functions during their use phase.[204] However, a major share of the total plastics market are plastic packaging, single-use plastics and microplastics such as microbeads, and these are indeed causing a great environmental impact and are, for that purpose, part of the European strategy for plastics.[113,205] This problem could be partially solved by recycling these wastes, since plastics are completely recyclable. However, nowadays only a small amount of these wastes is effectively recycled: for example, in 2014, in Europe, only 29.7 % of plastic wastes were recycled.[95] Therefore, there is the need of alternative, sustainable, and more biodegradable/renewable plastics. Within this scenario, one of the most cost effective and sustainable methods to alleviate this dual challenge of reducing CO_2 emissions and plastic accumulation is through culturing of such microorganisms that are capable of fixing atmospheric CO_2 along with the production of easily degradable biopolymers.

Polyhydroxyalkanoates (PHAs) are bio-polyesters accumulated by different bacterial cells under the form of granules inside the cytoplasm. PHAs are completely biodegradable and are mainly produced from renewable sources such as organic acids or wastes.[113,144,206] PHAs have physical properties similar to those of polyethylene,[143,206] and can replace the common plastics in several applications, such as packing films, disposable bulk materials and paper coatings. Moreover, microbial PHAs production can be carried out by using mixed microbial cultures (MMCs). Volatile fatty acids (VFAs) are the direct metabolic precursors of PHA.[43,144] To reduce further the total cost of PHA production, VFAs can be produced from wastes or biomass through, for example, dark fermentation (DF).[113,144,207] Nevertheless, the VFAs composition

produced during dark fermentation could be influenced by many factors (such as inoculum, feeding, pH, temperature, etc.) which difficult to obtain always the same VFAs composition and consequently the same PHA polymer.

Electro synthesis (ES), MES or photocatalytic reduction can be used to reduce CO_2 leading to the bio-production of short chain fatty acids such as acetate and butyrate.[201,208,209,210] Recently, Batlle-Vilanova et al.[211] showed the bioelectrochemical production of acetate and butyrate at a constant composition from CO_2 as the sole carbon source.

Starting from that work, here we are proposing the production of PHAs from CO_2 by a three steps process consisting in i. CO_2 reduction to acetate and butyrate through MES followed by ii. Extraction/Concentration of acetate and butyrate from the liquid broth; iii. PHAs production by using VFAs produced in step i as carbon source by a MMC. To the best of our knowledge, only few works reported the possibility of PHAs production starting from CO_2 as carbon source. In particular, the combination of MES with PHA production has been reported here for the first time. Moreover, an overall CO_2 conversion efficiency and C balance were also proposed for the first time.

5.2 Materials and methods

BES setup and operation

The bioelectrochemical system (BES) consisted in a two-chambered tubular reactor. The cathode and the anode were concentric and separated by a tubular cation exchange membrane (CMI-1875T, Membranes international, USA). An electrode surface of 320 cm^2 of commercial carbon cloth (Thickness 490 μm; NuVant's ELAT, LT2400W, FuelCellsEtc, USA), and 200 cm^2 of Ti-MMO (NMT® electrodes, South Africa) were uses as cathode and anode electrodes, respectively. An Ag/AgCl electrode (+0.197 V vs. SHE, model RE-5B, BASI, UK) was placed in the cathode chamber and used as reference electrode. The BES was operated in a three-electrode configuration with a potentiostat (BioLogic, Model VSP, France), controlling the cathode potential (working electrode) at -0.8 V vs SHE and monitoring the current demand.

Both anode and cathode were connected to external buffer tanks for the recirculation of liquid (5.8 L h^{-1}), and sampling of the liquid and gas. The net liquid volume of the cathode and the anode was 1300 mL and 1490 mL, respectively. The BES was thermostatically controlled at 38 ± 1 °C.

Experimental procedure

The BES was operated in batch mode. The anode and cathode compartments were filled with low-buffered mineral medium prepared based on a modified ATCC1754 PETC medium[208]. The

only carbon source was CO_2 (99.9 %, Praxair, Spain), which was periodically supplied (every 1-4 days) to the BES by directly sparging CO_2 gas into the anode and cathode compartments. The cathode was initially inoculated with 20 mL of an enriched carboxydotrophic mixed microbial culture (suspended biomass ~0.1 g L^{-1}) taken from a syngas fermentation reactor dominated by *Clostridium spp.*, which was able to produce a mixture of VFA and alcohols.[212] Multiple tests were carried out in the BES to produce a mixture of VFA and alcohols from CO_2, in which butyrate and acetate were obtained as the main final products.[211] The amount of CO_2 provided and consumed in the BES were quantified to calculate CO_2 concentrations and transformation efficiencies. Liquid and gas-phase samples were collected regularly (every 1-4 days) to analyze the production of VFA and alcohols, and to characterize the gas composition of the headspace, respectively.

BES Analyses and calculations

- Liquid and gas analyses

VFA and alcohols in the liquid phase were analyzed with an Agilent 7890A (Agilent Technologies, US) gas chromatograph (GC) equipped with a DB-FFAP column and a flame ionization detector (FID).

The pressure of the gas phase in the reactor was measured before sampling. Then, gas samples were collected from the reactor with a glass syringe and analyzed immediately in a second channel of the Agilent 7890A GC equipped with an HP-Molesieve column and a thermal conductivity detector (TCD). Partial pressure of H_2 (pH_2) and CO_2 (pCO_2) were calculated from the total pressure measured in the biocathode, and the composition of the gas phase. The concentration of dissolved H_2 and CO_2 (mM) was calculated using Henry law and assuming CO_2 saturation and lack of H_2 immediately after each gas supply. The total amount of CO_2 in the reactor was then corrected according to reactor liquid volume. CO_2 transformation efficiency was calculated as the ratio between the amount of carbon (C) found in products, and the total amount of C in form of CO_2 depleted.

Extraction and concentration of VFA

Acetate and Butyrate were separated and concentrated by liquid membrane extraction. Hollow fiber membranes (S6/2, Membrana, Germany) made of polypropylene (1.3 m length, 180 μm inner diameter, 0.2 μm pore diameter and 400 μm thickness) were used. Membrane pores were impregnated with an organic solution of dodecane containing 6 % dodecanol.[211] The excess of solvent was removed by immersion of the fiber in reagent water. The fiber was immersed into the production broth, and 30 mL of an acceptor phase (0.1 M NaOH) were recirculated through

the lumen of the fiber. The concentration of products in the acceptor phase was measured for a period of 95 h.[211]

PHA production

Inoculum and media

The enrichment of PHA-producing bacteria was performed by using a MMC constituted by an activated sludge (8 g Total Suspended Solids - TSS - L^{-1}) collected from the secondary sedimentation tank of a wastewater treatment plant (5.2×10^5 equivalent inhabitants) located at Peschiera Borromeo (Milan, Italy). The substrate used to select PHA-accumulating bacteria was prepared from the effluent rich in VFA (final concentration ≈400 mmole C L^{-1}), obtained during MES from CO_2 and diluted by the standard mineral medium[213] to have a final concentration of 40 mmole C L^{-1}. The substrate used to accumulate PHA was prepared by VFA solution with a standard mineral medium described above (final VFA concentration 200 mmole C L^{-1}) but without the addition of NH_4Cl and thiourea, in order to keep the same ratio between C and the other elements as in the selection medium. All substrates used for PHA production were sterilized after preparation.

PHA-producing bacteria enrichment

The enrichment in PHA-producing bacteria was performed in a Sequencing Batch Reactor (SBR) with a working volume of 1000 mL, applying an aerobic dynamic feeding (ADF) strategy.[113] In brief, the SBR cycle length was of 12 h, consisting of four discrete phases: i. influent filling (4 min), ii. aeration (675 min), iii. settling (40 min) and iv. withdrawal of the exhausted effluent (5 min) with 1 day of Hydraulic Retention Time (HRT) and 5 days of Sludge Retention Time (SRT), keeping the temperature at 25 °C and the pH at 8.8, this latter controlled by automatically set-point, adding 1 mole L^{-1} HCl. Aeration and agitation were provided by supplying air at 6 L min^{-1} and stirring set at 110 rpm. Pumping, aeration and stirring were automatically controlled. Samples were taken along the cycle once per SRT.

The enrichment process lasted for 29 days. The selection trend was monitored by determining the duration of the feast phase, achievable by using the dissolved oxygen (DO) concentration in the selection media,[66] measured by an optical probe (FDO 925, WTW, Germany). The feast to famine ratio (F/F ratio) was calculated as the ratio between the lengths of the two phases in hours (h). As reported by Valentino et al.[45], for a correct selection of PHAs-storing bacteria the F/F ratio had to be equal or less than 0.33.

To perform the selection of PHA-accumulating bacteria, 500 mL of activated sludge were used as inoculum fed, for each cycle, with 500 mL of substrate. Organic Loading Rate (OLR) was

kept close to 40 mmole C L^{-1} d^{-1} and the C/N/P ratio was of about 100:10:1 mmole C/ mmole N/ mmole P. For each SRT, samples were taken in order to assess the performance of the enriched culture.

PHA accumulation procedure

The ability of the MMC to accumulate PHA was assessed by fed-batch assays carried out in a 500 mL working volume glass reactor, with continuous aeration and stirring. These assays consisted in feeding the substrate to 250 mL of enriched culture (at least 3 SRTs from the beginning of the selection)[106] adopting a pulse wise feeding method. The assays were monitored in continuous by measuring the concentration of the DO in the accumulation media.[66] In particular, substrate was fed to the reactor when DO showed a strong increase.[66] Total C dosed was calculated taking into account that the ratio of the carbon to the microorganisms had to be the same of that inside the selection reactor. The assays were stopped when no DO variation followed the substrate feeding.

For the accumulation tests, the operating conditions adopted were those adopted in the selection reactor, i.e. temperature of 25 °C, aeration of 6 L min^{-1} and the stirring of 110 rpm.

The biomass from the selection process was submitted to accumulation tests using the same substrate used for the selection process but with a C concentration of 200 mmole L^{-1} and without ammonia salts addition, since it was reported that N limitation determined a greater conversion of carbon in PHA because of cell growth limitation[57]. During the fourth and the sixth SRT an accumulation test was performed in duplicate for each SRT.

Analytical methodologies

The substrates fed during the selection and accumulation processes were characterized in terms of soluble Chemical Oxygen Demand (COD), organic acids (OA) content (acetate, butyrate), nitrogen (N), ammonium ($N\text{-}NH_4^+$) and microelements content.

During the selection trials, every sample was also characterized in terms of pH, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), soluble COD, $N\text{-}NH_4^+$ content and PHA content. During accumulation trials, samples were taken continuously in order to measure TSS, VSS, soluble COD and PHA content.

Biomass concentration was calculated as VSS according to the standard methods.[66]

TSS and VSS were determined as reported by Valentino et al.[67]. The COD and the $N\text{-}NH_4^+$ content (filtered at 0.45 μm) were determined using cuvette test kits (Macherey-Nagel, Germany).

PHA were determined by GC-MS using a method adapted from Serafim et al.[57]. Lyophilized biomass was incubated for methanolysis in a 20 % v/v H_2SO_4 in MeOH solution (1 mL), and then extracted with chloroform (1 mL). The mixture was digested at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was extracted and analyzed by GC-MS as described previously.[113,144]

Calculations

The PHA content in cells was reported as percentage of VSS on a mass basis:

$$PHA = (g\ PHA\ /100g\ VSS)$$

considering VSS to be constituted by both active biomass (X) and PHA.[66] PHA was converted into C basis according to its molecular weight: 0.046 mole C g^{-1} HB monomer.[45] Acetate and butyrate were considered as HB precursors.[66]

X was calculated on a C mole basis considering that 1 g of active biomass contains 44.2 mmole C.[66] Specific substrate consumption rate (q_C, mmole C $mmole^{-1}$ C_X h^{-1}) was determined as the ratio between the amount of soluble COD consumed, and the time needed to deplete it per unit of active biomass. Specific PHA storage rate (q_{PHA}, mmole C_{PHA} $mmole^{-1}$ C_X h^{-1}) was determined as the ratio between the amount of PHA stored and the time needed to deplete the soluble COD per unit of active biomass. Specific growth rate (q_X, mmole C_X mmole-1 C_X h^{-1}) was determined as the ratio between the amount of active biomass growth and the time needed to deplete the soluble COD per unit of active biomass, as reported by Valentino et al.[45].

For the SBR, the PHA storage yield (mmole C_{PHA} $mmole^{-1}$ $C_{cons.}$) was calculated as the ratio between the amount of PHA accumulated and the amount of soluble COD depleted. Again the growth yield (mmole C_X $mmole^{-1}$ $C_{cons.}$), was calculated as the ratio between the new active biomass produced and the amount of soluble COD depleted, as reported by Valentino et al.[45].

In the accumulation batches, the specific rates and yields were calculated as described before, for each pulse, except for the specific growth rate and the growth yield that were not calculated because of the absence of growth during the accumulation trials. Moreover, PHA storage yield on carbon fed was considered in the accumulation test (mmole C_{PHA} $mmole^{-1}$ C_{in}), calculated as the ratio between the amount of PHA accumulated and the amount of soluble COD fed. In order to compare different accumulation tests, the average values of the first three pulses and for each parameter were considered.

5.3 Results and discussion

An integrated advanced process based on a bioelectrochemical system coupled to a PHA accumulation process was proposed to produce bioplastics from CO_2, as the sole carbon source. In the first step, CO_2 was bioelectrochemical transformed to acetate and butyrate while in the second step these VFA were accumulated as 3-hydroxybutyrate (3-HB). Each step was analyzed to evaluate the overall efficiency of the process.

Bioelectrochemical acetate and butyrate production from CO_2

The bioelectrochemical system was operated for 70 days. Several tests were carried out to drive the C-compounds production to butyrate instead of acetate. Figure 5.1 shows the evolution of CO_2 consumed, total amount of carbon in products and the main compounds obtained (i.e. acetate and butyrate) over time, in one representative test. Carbon consumed and the total amount of carbon found in products increased progressively following a linear trend. The amount of carbon in products increased at the rate of 6.68 ± 0.38 mmol of C day^{-1} (r^2=0.966; $p<0.05$, n=12), compared to 9.14 ± 0.21 mmol of C day^{-1} (r^2=0.977, $p<0.05$, N=12) of total CO_2 consumption. CO_2 transformation efficiencies or the amount of CO_2 that is captured in the form of products during MES have not been typically examined in previous studies. According to the values obtained in the present study, CO_2 fixation efficiency into products was 73.0 %. Acetate increased at the beginning of the test and then remained stable at 39.5 ± 4.8 mmol C-Acetate because it was partly used for butyrate production. Butyrate increased along the test up to 103 mmol C-Butyrate on day 51. The main reactions that supposedly took place during MES in the present work were described previously by Batlle-Vilanova et al.[211]. Bioelectrochemical H_2 production could work as a trigger for the subsequent reactions where the presence of CO_2 and H_2 into the bioreactor would initiate a series of reactions, consisting of: (i) homoacetogenesis of CO_2 and H_2 to acetate, (ii) reduction of acetate to ethanol at the expenses of H_2, and (iii) chain elongation (reverse β-oxidation) of acetate and ethanol into butyrate.

At the end of the process, VFAs produced from CO_2 were extracted from the liquid broth by liquid membrane extraction, which resulted in a current with a high concentration of butyrate versus acetate (molar ratio 16.4).[211]

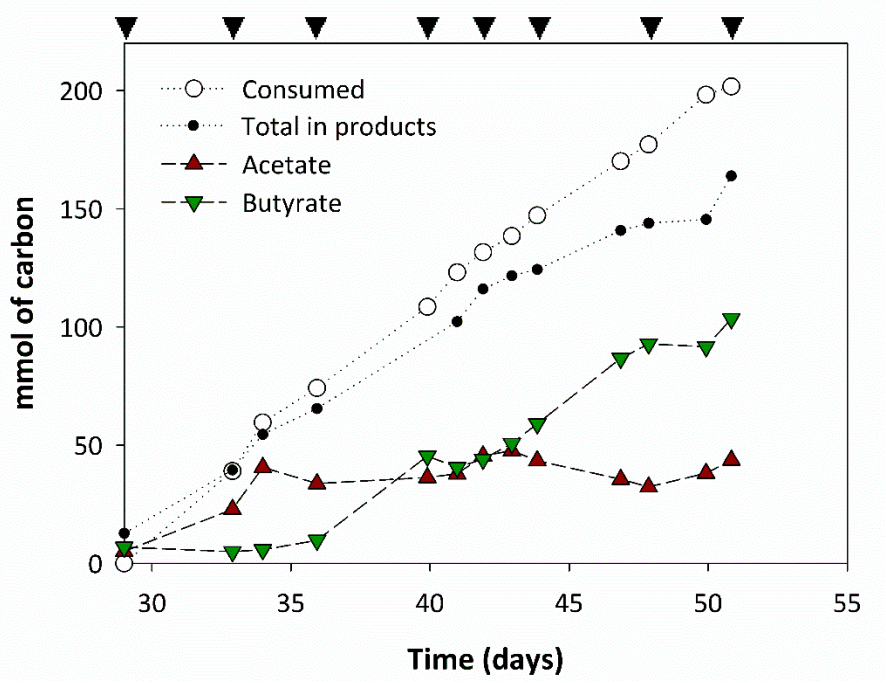

Figure 5.1 *Carbon dioxide (CO_2) consumption and accumulation of carbon in products as function of time during microbial electrosynthesis (MES) in the biocathode of the bioelectrochemical system (BES). Data adapted from test 2 of Batlle-Vilanova et al.*[211]*. Black inverted triangles at the top of the graph indicate CO_2 supply*

Polyhydroxyalkanoates (PHA) selection and accumulation

The effluent of the extraction membranes test after a pH adjustment was used as carbon source for the PHAs selection and accumulation. From the beginning of the test, the selection process showed a feast/famine ratio (F/F) under 0.14 (Figure S1) with a following trend between 0.08-0.02. The ratio obtained was in line with the correct feast/famine ratio for the growth of PHAs accumulating bacteria.[45] As indicated in table 5.1, the maximum PHA content at the end of the feast phase was 19.3 g PHA 100g VSS^{-1} where the entire polymer produced was constituted by PHB (Table 5.1). Analyzing the results obtained in table 5.1, the specific substrate consumption rate obtained was 0.65 mmole C $mmole^{-1}$ C_X h^{-1}, the specific PHA storing rate was 0.51 mmole C_{PHA} $mmole^{-1}$ C_X h^{-1} and the specific growth rate was 0.18 mmole C_X $mmole^{-1}$ C_X h^{-1}. For what concern the PHA storage yield on substrate consumed, 0.79 mmole C_{PHA} $mmole^{-1}$ $C_{cons.}$ was found while for the growth yield expressed on substrate consumed 0.28 mmole C_X $mmole^{-1}$ $C_{cons.}$ was recovered. The comparison between both yields showed how the bacterial used the carbon source for PHA storage instead of growth, confirming that the selection process was conducted in the right way.[57] Moreover, the others parameters typically used to determine the

selection in PHAs-storing bacteria trends were similar with those reported previously (Table 5.1).

Table 5.1 *Comparison between the performances of the PHA-storing culture selected in this study and in the literature*

Substrate	**PHA content**[a] (g PHA 100 g^{-1} VSS)	**Polymer composition (ΔHB/ΔHV)** (%, w/w)	**$-q_C$**[b] (mmole C mmole^{-1} C_X h^{-1})	**q_{PHA}**[c] (mmole C_{PHA} mmole^{-1} C_X h^{-1})	**q_X**[d] (mmole C_X mmole^{-1} C_X h^{-1})	**PHA Yield**[e] (mmole C_{PHA} mmole^{-1} $C_{cons.}$)	**Growth Yield**[f] (mmole C_X mmole^{-1} $C_{cons.}$)	**Ref.**
Acetate +Butyrate	19.3	100/0	0.65	0.51	0.18	0.79	0.28	This study
Acetate	42±3	100/0	0.5±0.0	0.2±0.0	0.14±0.0	0.4±0.0	0.25±0.01	[113]
Acetate	53	100/0	3.2	2.1	-	-	-	[214]
Butyrate	14.5	100/0	0.10±0.01	0.071±0.002	-	0.45	0.3	[213]
Acetate	17.5	100/0	1.10±0.05	0.41±0.02	-	0.49	0.3	[57]

[a]PHA accumulated at the end of the feast phase referred to VSS;
[b]specific substrate consumption rate;
[c]specific PHA storage rate;
[d]specific growth rate;
[e]PHA storage yield expressed on substrate consumed;
[f]Growth yield expressed on substrate consumed.

After the microbial culture selection, two accumulation tests were conducted as described in M&M, in order to evaluate the maximum PHA storing capacity of the selected culture. For the first accumulation test, 200 mL of the selected culture were sampled after the 4th sludge retention time (SRT) while for the second accumulation test the culture was sampled after the 5th SRT. During the test, the microbial culture was fed in pulse-wise mode adding only the carbon medium solution adjusted with microelements but excluding any nitrogen source. This was done, because it had been reported that N limitation lead to a greater conversion of carbon in PHA because of cell growth limitation,[57] therefore decreasing the growth yield while increasing the PHA yield. The accumulation trends, i.e. dissolved oxygen (DO_2) versus PHA accumulation versus substrate consumption, were similar in both accumulation tests (Figure 5.2a and 5.2b). In particular, as reported in figure 5.2a and 5.2b just after four pulses (≈ 150 h), the DO_2 did not show a significant decrease due to the PHA accumulation, i.e. less VFAs consumption[57].

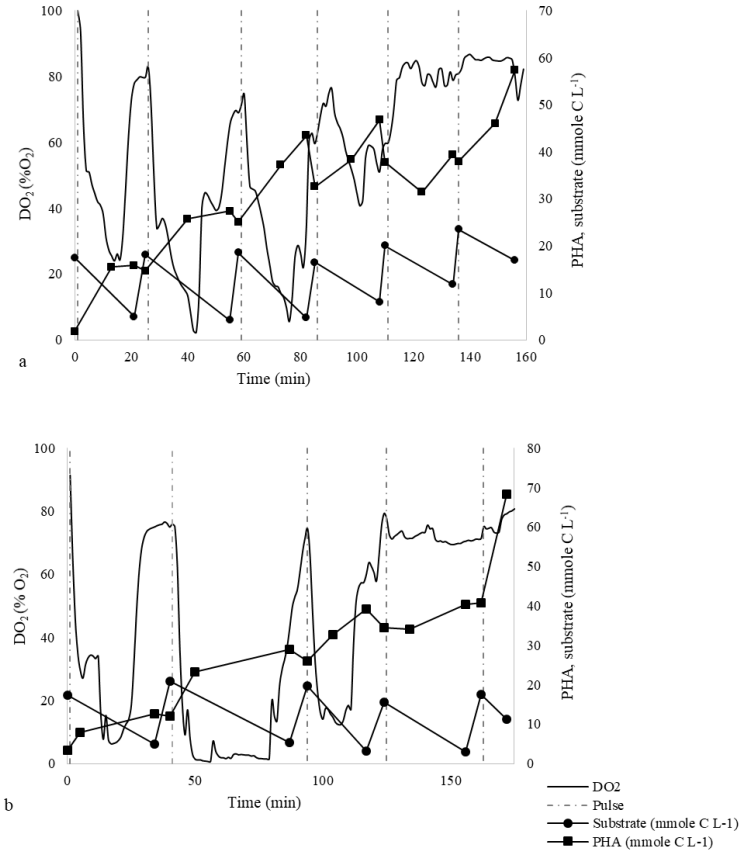

Figure 5.2 *DO_2, substrate and PHA trend during the accumulation test 1 (a) and the accumulation test 2 (b). Substrate includes the total concentration of carbon (C) supplied in the form of volatile fatty acids (VFA), obtained by MES from CO_2*

As indicated in table 5.2, 74.4 ± 0.6 g PHA $100g^{-1}$ VSS and 69.01 ± 0.4 g PHA $100g^{-1}$ VSS were produced during the first and the second accumulation test respectively. In particular, the PHA storage yield on substrate fed got during the accumulation tests were 0.66 ± 0.17 and 0.77 ± 0.18 (mmole C_{PHA} $mmole^{-1}$ C_{in}) respectively. These data were in line with 0.6 mmole C_{PHA} $mmole^{-1}$ C_{in} reported by Johnson et al.[214] (Table 5.2). For what concern other parameters such as the PHA storage yield on substrate consumed (Table 5.2), the results obtained where higher than the data reported previously[113].

Table 5.2 *Comparison between PHA accumulation trials performed in this study and tests reported in literature*

Test	**Substrate**	**PHA content**[a] (g PHA 100 g^{-1} VSS)	**Polymer composition (ΔHB/ΔHV)** (% w/w)	**-q_C**[b] (mmole C $mmole^{-1}$ C_X h^{-1})	**q_{PHA}**[c] (mmole C_{PHA} $mmole^{-1}$ C_X h^{-1})	**PHA Yield**[d] (mmole C_{PHA} $mmole^{-1}$ $C_{cons.}$)	**PHA Yield**[e] (mmole C_{PHA} $mmole^{-1}$ C_{in})	**PHA Productivity** (g PHA L^{-1} d^{-1})	**Total Biomass** (g VSS L^{-1})	**Ref.**
Acc. 1	Acetate +Butyrate	74.44±6	100/0	0.64±0.37	0.57±0.38	0.87±0.19	0.66±0.17	15.51±4.55	1.51±0.05	This study
Acc. 2	Acetate +Butyrate	69.01±4.1	100/0	0.67±0.20	0.78±0.37	1.12±0.20	0.77±0.18	11.35±4.03	1.37±0.04	This study
Acc.	Acetate	82.4±5.8	100/0	0.44±0.03	0.29±0.01	0.68±0.04	-	0.9	0.54	[113]
Acc.	Acetate	89 (g PHA 100 g^{-1} TSS)	100/0	-	1.4	-	0.6	-	-	[214]

[a]PHA accumulated at the end of the test referred to VSS;
[b]specific substrate consumption rate;
[c]specific PHA storage rate;
[d]PHA storage yield expressed on substrate consumed;
[e]PHA storage yield expressed on substrate fed.

Moreover, according with the table 5.2, the PHA yield on C consumed was of 1.12 ± 0.2, i.e. 100 %, in line with data reported in the literature[73,215]. This result showed that the microbial population converted all C consumed in PHA without the production of other compounds, i.e. glycogen, demonstrating the good microbial population selection occurred during the selection process. Furthermore, the PHA polymer composition obtained during the selection and the accumulation processes was reported; according with data achieved, only polyhydroxybutyrate (100 % PHB) was obtained. The polymer composition obtained was in line with the PHB production pathway proposed previously by Reis et al.[43] when acetate and butyrate are used as carbon sources by MMC to produce P(3HB). In this possible pathway proposed for MMC, when acetate is used as carbon source, two units of acetyl-CoA are condensed by 3 ketothiolase (PhaA) to form acetoacetyl-CoA, which is reduced by acetoacetyl-CoA reductase (PhaB) to (R)-3-hydroxybutyryl-CoA, which is subsequently incorporated into the polymer chain as (3HB) by PHA synthase (PhaC).[43] Butyrate can be directly converted into to (R)-3-hydroxybutyryl-CoA and incorporated into the polymer as (3HB) as described above. The possibility to produce selectively VFAs (acetate or butyrate) or alcohols from a greenhouse gases (CO_2) as feedstock by changing the applied voltage to the MES,[211] it could be useful for controlling the subsequent PHAs polymer composition. In fact, the VFAs composition made available to the PHA-storing microorganisms influences the PHA composition, which in turn affects their physical and mechanical properties.[206] For this reason, it would be possible to create a specific VFAs mix via MES with the goal of obtaining a specific PHA polymer to be used for a precise application. Our results confirmed that controlling VFAs production by MES instead of dark fermentation where is difficult to control VFAs production in depth, it could be possible to have a stable PHA polymer composition.

Overall carbon and energy balances

An overall conversion of CO_2 to PHA, i.e. C balance, was calculated taking into account the whole process proposed in this work. Two efficiencies were calculated, one based on the C_{CO2} consumed; i.e. the C_{CO2} consumed by bacteria, and the other one on the total C_{CO2} inlet in the entire system. During the MES process the yield of acetate and butyrate produced from C_{CO2} consumed, (mmole C_{VFA} mmole^{-1} C_{CO2}) was of 0.70 ± 0.05 mmole C_{VFA} mmole^{-1} C_{CO2} with a maximum of 0.78 ± 0.03 mmole C_{VFA} mmole^{-1} C_{CO2}. During PHA accumulation all the C provided was converted to PHA. For this reason, the overall average PHA yield (expressed as mmole of C) produced on C_{CO2} consumed (mmole C_{PHA} mmole^{-1} C_{CO2}) was 0.70 ± 0.05. For what concern the VFA yield on total C_{CO2} inlet, 0.53± 0.08 mmole C_{VFA} mmole^{-1} $C_{CO2\text{-}in}$ was

obtained by the MES system. In this case the overall efficiency was calculated taking into account the PHA yield (expressed as mmole of C) on carbon fed in the PHA bioreactor reported in table 5.2, i.e. 0.77 ± 0.18 mmole C_{PHA} mmole^{-1} C_{in}. The average overall efficiency was 0.41 ± 0.06 mmole C_{PHA} mmole^{-1} $C_{CO2\text{-}in}$ with a maximum of 0.50 ± 0.07 mmole C_{PHA} mmole^{-1} $C_{CO2\text{-}in}$. Figure 5.3 shows schematically the efficiency obtained in terms of C conversion, considering 1 kg of carbon as C_{CO2} fed to the entire system. Balance reported indicated that 0.41 kg of C-PHA could be obtained.

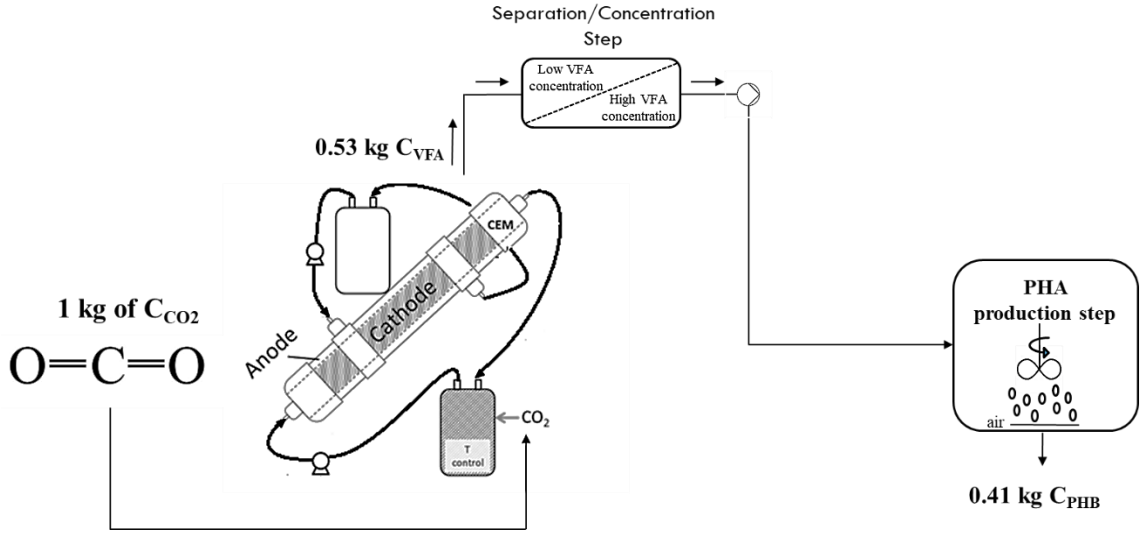

Figure 5.3 *Schematic representation of the system proposed in this work compared with the carbon efficiency conversion by MES and PHA production. CEM=cation exchange membrane*

The concept presented in this work is framed within the concept of power-to-gas[216] or power-to-products. Technological assessment regarding process feasibility of these technologies is crucial for its further development. Among others, the amount of energy consumed per unit of product that is obtained is a good indicator to evaluate the efficiency of the process.[217] Regarding the energy consumption of the process, it has to be considered that bioelectrosynthesis consumed electricity in form of electrons as reducing power source. The average cell voltage that resulted from applying a half-cell potential of -0.8 V vs SHE in the cathode chamber, was of 3.63 ± 0.18 V. The average energy demand of the BES was calculated considering only the electron requirement, which resulted in 7.84 ± 0.01 Wh day^{-1}. Comparing this value to the carbon production rate, it can be found that the energy supply needed for CO_2 transformation into VFA by MES was 1.18 Wh mmole^{-1} C_{VFA}. This value was just 3 times higher than the theoretical sum of the thermodynamic values ($\Delta G_r^{o'}$), i.e. 0.30 Wh mmole^{-1}

C_{VFA}, calculated taking in to account the 4 reactions involved in producing VFAs during the MES system.[141] Given the fact that PHA accumulation phase require O_2 for the process, according to the data reported by Gurieff et al.[218], the energy requirement for PHA accumulation is 0.17 Wh mmole^{-1} C_{PHA}. For this reason, the overall energy consumption obtained in the present study was of 1.35 Wh mmole^{-1} C_{PHA}.

5.4 Conclusions

This study puts forward a new resilience and sustainability biorefinery concept where the reduction of CO_2 into VFAs (acetate and butyrate) is integrated with its subsequent consumption and selective transformation into bioplastic (3-hydroxybutirate) by PHA accumulation. Moreover, the process allows a stable production of VFAs and consequently, a stable PHA polymer composition. The mass and energy balances assessment concluded that 0.41 kg of C-PHA could be obtained per kg of C-CO_2 with an energy demand of 1.35 Wh mmole^{-1} C_{PHA}.

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Supporting information

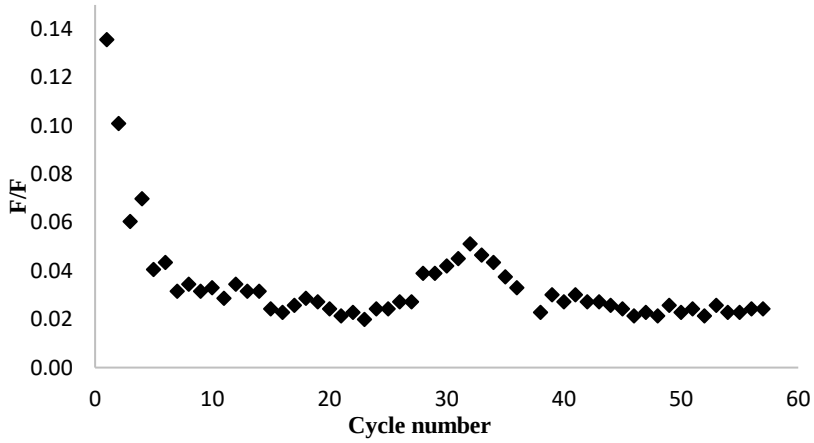

Figure S1 *Feast famine ratio (F/F) trend during all the selection*

6. Non-ionic surfactants as a pretreatment to optimize PHA extraction from mixed microbial cultures by using DMC

Colombo, B., Pereira, J., Ventura, S., Adani, F., Serafim, L.S.

6.1 Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters synthesized by many bacterial species as intracellular reserves of carbon and energy. Their biodegradability and thermochemical properties make them suitable as a greener alternative to conventional plastics.[43] Despite their potential PHAs high production costs still limit their use.

The use of mixed microbial cultures (MMCs) instead of pure cultures and agro-industrial wastes as substrates has been used as a way of reducing such expenses, however the downstream processing still represents a considerable percentage of the total production costs. Nowadays the extraction process is known to count for about the 30-50 % of the total costs,[59,64] due to extensive use of non-recyclable (and sometimes highly toxic) chemicals/materials and due to the high energy consumption[89].

Downstream strategies consist mainly in PHAs granules extraction from the bacterial cells, in some cases preceded by pre treatments in order to get a better recovery yield and/or followed by a purification step to get a higher purity; the ideal method should lead to a high purity and recovery level at a low production cost.[90]

The extraction procedures currently adopted on pure microbial strains can be divided into two classes: PHAs solubilisation/recovery with organic solvents and the dissolution of Non-PHA Cell Mass (NPCM) with chemicals (i.e. acids, alkalis and surfactants) or enzymes.[59,91]

However, both the approaches present several disadvantages:

- Chlorinated organic solvents, such as chloroform and dichloromethane, are the best performing, but since they create hazards for the operators and the environment, they still do not represent a good choice at an industrial level. Other more sustainable solvents have been tested (i.e. propylene carbonate, ethyl acetate, methyl isobutyl ketone), reporting good results in terms of purity and recovery yields, but negative impact on the molecular weight of the final polymer, due to the high temperature required. Moreover, other “green” alternatives, such as ionic liquids and supercritical fluids, are still too costly to represent a valid substitute.[59]

- Concerning NPCM dissolution with chemicals, at the beginning nonselective dissolution systems were adopted, mainly represented by alkalis (i.e. NaOH and NaClO) and acids digestion, which if too concentrated can determine a degradation of both NPCM and PHAs, thus reducing the recovery yield and lowering PHAs molecular weight. These methods were then replaced by selective dissolution chemicals, including anionic (i.e. sodium dodecyl sulphate - SDS), cationic (i.e. hexadecyltrimethylammonium bromide - CTAB), non-ionic surfactants (i.e. Tween 20 and Triton X-100) and proteolytic enzymes. For what concern the use of surfactants, all of them can be directly applied to the wet microbial culture, thus avoiding the expensive dewatering step, and they do not affect the properties of the final product. On the other hand, to work properly, a huge amount of surfactant is needed, determining high wastewater production to be necessarily recovered in order to contain the costs. Another drawback of this procedure is the low purity of the extracted PHAs, that often require a final purification step.[59,91]

As above reported, several works have been developed to propose and optimize solutions for PHAs extraction from pure cultures, while MMCs are often overlooked mainly because of their heterogeneity and resistance to cell hydrolysis, thus determining the need of greater efforts in PHAs recovery.[89]

Samorì et al.[89] reported an innovative PHAs extraction procedure from MMCs by proposing the use of dimethyl carbonate (DMC), a non-polar solvent with good miscibility with water, readily biodegradable, and non-toxic[219].

Starting from these results, in order to increase the extraction yield and maximize purity, a biomass pre treatment step could be considered. This pre treatment can include the use of surfactants.

Non-ionic surfactants are amphiphilic molecules whose effect on cell aggregation and membrane permeability was already reported, as well as their use as extraction agents in biological processes.[220,221,222]

In this work, a biomass pre treatment using non-ionic surfactants is proposed as a way of increasing the efficiency of DMC in PHAs extraction from a mixed microbial culture selected on fermented agro-industrial wastes.

6.2 Materials and methods

Materials

Microbial biomass

To develop the PHA extraction procedure a PHA-rich mixed microbial culture collected from an accumulation reactor fed with fermented agro-industrial wastes was used. The microbial biomass was centrifuged at 10,000 g for 15 min at room temperature, the supernatant was discarded, and the pellet washed three times with 0.9 % of NaCl solution. The final pellet was freeze-dried and kept in a desiccator for the determination of polymer content by gas chromatography.

Extractive agents

The reagents used in this study to perform the PHA extraction from a mixed microbial culture were: polyoxyethylene sorbitan monolaurate (Tween® 20), polyethylene glycol dodecyl ether (Brij® L4), dimethyl carbonate (DMC), chloroform, all supplied by Sigma-Aldrich (Darmstadt, Germany), and polyethylene glycol tert-octylphenyl ether (Triton™ X-114) supplied by Acros Organics, Thermo Fisher Scientific (Waltham, MA, USA).

Methods

Optimization of the extraction procedure

The extraction procedure developed in this work consisted of two main steps, the first step was represented by a selective dissolution of the non-PHA cell mass (NPCM) by using non-ionic surfactants, while the second step was represented by a PHA solvent extraction with dimethyl carbonate (DMC) from the pre treated microbial biomass.

Briefly, to start the optimization of the extraction procedure, during the first step 0.1 g of lyophilized biomass were suspended in a specific amount of non-ionic surfactant at 60 °C, under stirring at 80 rpm. The pre treated biomass was centrifuged at 5,500 rpm for 30 min at 25 °C, the supernatant was discarded and the pellet was washed three times with 5 ml of deionized water, 5 ml of EtOH and 5 ml of deionized water. After having discarder the supernatant, the pellet was then left to dissolve in a specific amount of DMC. Finally, the mixture was vacuum filtrated with glass microfiber membranes (0.45 µm of porosity), and the DMC was left to evaporate. The obtained residue was weighted and the PHA content estimated based on the percentage of PHA present in the initial biomass.

During this study the extraction procedure was submitted to an optimization of the operating conditions by testing different values of the parameters reported in table 6.1.

Table 6.1 *Parameters tested during the optimization of the developed extraction procedure*

1^ step (non-ionic surfactant)				
Contact time (h)	2	4	8	
Surfactant	Tween® 20	Triton™ X-114	Brij® L4	
Solid/liquid ratio (g mL^{-1})	0.03	0.0625	0.095	0.16
Surfactant concentration (mM)	100	150		
2^ step (DMC)				
Temperature (°C)	25	65	90	
Solid/liquid ratio (g mL^{-1})	0.025	0.075		

The parameters that allowed to obtain the best results in terms of polymer recovery yield were used to define the optimized protocol proposed at the end of this work.

Solubilisation tests

To minimize the risk of losing PHA by solubilizing it during the biomass digestion with non-ionic surfactant, some solubilisation tests were performed to confirm that PHA would not solubilize in the desired extractive agent. The tests were performed by mixing 1 g of industrial P(3HB) (Sigma-Aldrich, Darmstadt, Germany) with 10 mL of surfactant (Tween® 20, Triton™ X-114, Brij® L4) at 60 °C for 4 h, with gentle mixing. The liquid fractions were then analysed in FT-IR to evaluate the possible presence of the biopolymer.

Control tests

In order to evaluate the effectiveness of the optimized extraction procedure, two control tests were performed, one with DMC and one with chloroform. In particular, for the DMC test the procedure reported by Samorì et al.[89] was followed, while for the chloroform test the extraction method reported by Duque et al.[66] was followed.

Analytical methods

- Gas chromatography

Gas chromatography with a coupled flame ionization detector (GC-FID) was used to determine the PHA concentration in the biomass sample, using a chromatograph Clarus 480 from Perkin Elmer equipped with a column SGE BP20 (WAX) (length: 60m: inner diameter: 0.32mm; film thickness: 0.5µm) and using a method adapted from Lemos et al.[213]. The lyophilized biomass was incubated at 100 ºC for 3.5 h with heptadecane solution as internal standards, dissolved in chloroform (1:1) and 20 % acidic methanol. After digestion, the organic fraction of each sample was extracted and injected in the GC-FID. The HB and HV monomers concentration were calculated using P(HB-co-HV) (88 %/12 %) standards (Sigma-Aldrich, Darmstadt, Germany). The column temperature started at 50 ºC and was firstly ramped up to 100 ºC by 16 ºC min^{-1}

and followed by a second ramp of 9 ºC min^{-1} until reaching 220 ºC and kept and this temperature for 3 min.

- Fourier-transform infrared spectroscopy (FT-IR)

The PHA films and other substances extracted were analysed by FT-IR spectroscopy (PerkinElmer, Waltham, MA, USA) by adopting the following conditions: spectral range of 4000-500 cm^{-1} to allow the confirmation of the functional groups presented in the polymer, and 64 or 32 scans, respectively for solid or liquid samples. To analyse the extracted polymer, the homogenised sample was inserted above the crystal and then squeezed with an equipped press.

Calculations

The PHA recovery yield of the overall process was calculated using the following equation:

$$PHA\ recovery\ yield\ (\%) = \frac{m\ PHA\ (g)}{PHA\ biomass\ (\%) \times m\ biomass\ (g)}$$

where *m PHA* is the mass of the extracted polymer at the end of the process, *PHA biomass* is the percentage of PHA found in the lyophilized biomass and *m biomass* is the mass of biomass weighted for the extraction process.

6.3 Results and discussion

After having determined the PHA content of the microbial biomass used in this work, that was around the 50 % of the cell dry weight, in order to develop the extraction procedure, the method proposed by Samorì et al.[89] based on the use of DMC as PHA extractive agent from mixed microbial cultures was performed as a starting point. Three different extraction temperatures were tested (room T, 65 °C and 90 °C), by suspending 0.1 g of lyophilized biomass in DMC and keeping the same solid to liquid ratio (0.025 g mL^{-1}) tested by Samorì et al.[89]. It was observed that by adopting the highest temperature, the maximum polymer recovery yield was reached, equal to 38.42 ± 0.77 %. In fact, also Samorì et al.[59], by using DMC to extract PHA from a pure culture, observed that the increase of the temperature can positively affect PHA extraction.

Even if the highest temperature allowed to obtain the best result, the polymer recovery yield was about 20 % lower than what reported by Samorì et al.[89] on MMCs in the same thermal condition. In order to improve the recovery yield, before PHA extraction with DMC, a biomass pre treatment method was developed and applied.

The pre treatment consisted of a selective dissolution of the non-PHA cell mass (NPCM) with non-ionic surfactants. Three different non-ionic surfactants were tested: Tween® 20, Triton™ X-114 and Brij® L4. Before evaluating their digestive activity on NPCM, for the three surfactants a PHA solubilisation test was performed (see M&M), with negative results.

For each surfactant three different contact times were tested: 2 h, 4 h and 8 h, then followed by DMC's PHA extraction by adopting the condition above reported. It was surprisingly noticed that the pre treatment in any case allowed to increase the PHA recovery yield (Table 6.2). In particular, for each surfactant, it was observed that by increasing the time above 4 h, a reduction in the final polymer yield occurred. This phenomenon was probably due to a loss of a fraction of PHA inside the liquid surfactant phase, together with the NPCM. A contact time of 4 h was then adopted since it allowed to obtain in general the best recovery yield for all the surfactants tested.

Table 6.2 *Polymer recovery yield by testing three surfactants with three different contact times as a pre treatment of PHA extraction with DMC*

		Polymer recovery yield (%)		
Surfactant	**Concentration**	**2 h**	**4 h**	**8 h**
Tween® 20	150 mM	56.75	55.73	49.99
Triton™ X-114	5 % (w/w)	51.42	52.02	50.00
Brij® L4	5 % (w/w)	43.42	61.74	35.95
Control (DMC)			38.42 ± 0.77	

Even if Tween® 20 and Brij® L4 gave the best results, not so relevant differences were found among the three surfactants, and all of them were kept for the following optimization test.

For each surfactant four different solid to liquid ratios (S/L) were tested: 0.03 g mL^{-1}, 0.0625 g mL^{-1}, 0.095 g mL^{-1} and 0.16 g mL^{-1} by keeping the surfactant concentration at 150 mM (Table 6.3).

Table 6.3 *Polymer recovery yield by testing the three surfactants with four different S/L ratios at 150 mM as a pre treatment of PHA extraction with DMC*

		Polymer recovery yield (%)			
Surfactant	**Concentration**	**0.16 g mL^{-1}**	**0.095 g mL^{-1}**	**0.0625 g mL^{-1}**	**0.03 g mL^{-1}**
Tween® 20	150 mM	42.19	56.36	53.29 ± 2.39	57.50 ± 11.17
Triton™ X-114	150 mM	43.24	41.95	46.21 ± 8.66	44.37 ± 0.31
Brij® L4	150 mM	45.29	45.20	49.82 ± 3.17	54.50 ± 18.89
Control (DMC)			38.42 ± 0.77		

For the three surfactants, the lowest S/L ratio allowed to obtain the best recovery yield, while the highest S/L ratio resulted in almost the lowest amount of extract with lowest signal for PHA, as could be noticed from the FT-IR spectra of the extracted polymers. It was probably because the surfactants, reduced in amount by keeping the same quantity of biomass, acted mainly on the surface of the cells not helping in removing the remaining NPCM, as happened by adopting the other three S/L ratios. For this reason, the highest S/L ratio was discarded for the following optimization step.

In order to evaluate the effect of the concentration of the three surfactants, the three best S/L ratios were tested at 100 mM. The results obtained (Table 6.4) were in general similar or slightly lower with respect to the highest concentration tested. Only in correspondence of the S/L ratio of 0.095 g mL^{-1}, for Triton™ X-114 and Brij® L4 slightly higher results were achieved, while for Tween® 20 some problems occurred in the polymer recovery.

Table 6.4 *Polymer recovery yield by testing the three surfactants with three different S/L ratios at 100 mM as a pre treatment of PHA extraction with DMC*

		Recovery yield (%)		
Surfactant	**Concentration**	**0.095 g mL^{-1}**	**0.0625 g mL^{-1}**	**0.03 g mL^{-1}**
Tween® 20	100 mM	-	50.72 ± 3.63	45.19 ± 4.32
Triton™ X-114	100 mM	44.41	46.01 ± 0.98	34.15 ± 4.66
Brij® L4	100 mM	50.65	49.24 ± 0.38	48.05 ± 6.37
Control (DMC)			38.42 ± 0.77	

In any condition adopted (concentration and S/L ratio), Triton™ X-114 gave the lowest results in terms of polymer recovery yield with respect to the other two surfactants. For this reason, Triton™ X-114 was discarded while Tween® 20 and Brij® L4 were adopted as best digestive agents on NPCM. The best results were obtained at 150 mM and at the lowest S/L ratio, allowing to reach a final polymer recovery yield of 57 % and 55 %, respectively for Tween® 20 and Brij® L4. To underline the similarity between the extracted polymers with the selected conditions and the commercial polymer (P(3HB)), the obtained FT-IR spectra are shown in figure 6.1.

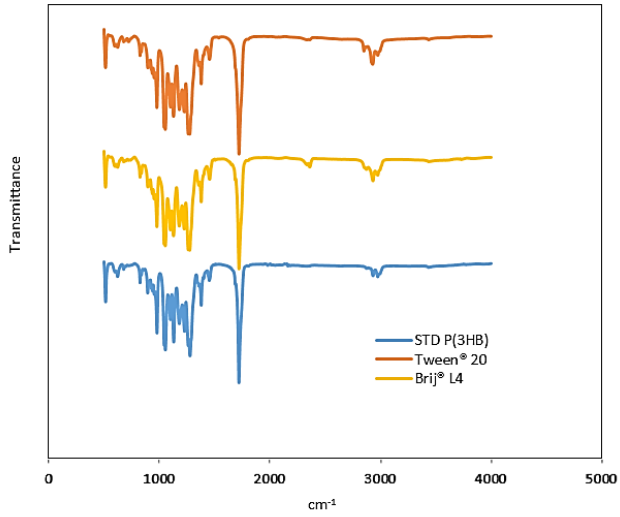

Figure 6.1 *FT-IR spectrum of commercial P(3HB) compared with the spectra of the polymers extracted with the best selected conditions*

In this work, the FT-IR was used to quickly detect the biopolymer presence in the extracted samples, by simply comparing the characteristics and peaks of the commercial P(3HB) spectrum, with those obtained for each sample's spectrum.[223] Regarding the industrial P(3HB) FT-IR spectrum, the main peak for PHA detection can be seen at ~1700 cm^{-1}, which represents the ester groups (C=O stretching vibrations). Between ~980 cm^{-1} to around ~1350 cm^{-1} other peaks can be observed corresponding to C-C bonds (~980 cm^{-1}), C-O-C symmetrical and unsymmetrical stretches (~1000 cm^{-1}), or CH_3 angular symmetrical deformation (~1350 cm^{-1}). The peaks at ~2900 cm^{-1} and ~2400 cm^{-1} correspond to water and carbon dioxide presence, respectively.[223]

Once optimized the pre treatment procedure, a last test was performed in order to evaluate if reducing the DMC amount similar polymer recovery yields could be obtained (Table 6.5).

Table 6.5 *Polymer recovery yield by testing a higher S/L ratio in the DMC step, keeping the best selected conditions for the pre treatment*

			Recovery yield (%)	
Surfactant	**Concentration**	**S/L ratio in the pre treatment**	**0.025 g mL^{-1}**	**0.075 g mL^{-1}**
Tween® 20	150 mM	0.095 g mL^{-1}	56.36	27.46 ± 5.45
Tween® 20	150 mM	0.0625 g mL^{-1}	53.29 ± 2.39	29.56 ± 0.92
Tween® 20	150 mM	0.03 g mL^{-1}	57.50 ± 11.17	34.40 ± 0.92
Brij® L4	150 mM	0.095 g mL^{-1}	45.20	27.10 ± 6.83
Brij® L4	150 mM	0.0625 g mL^{-1}	49.82 ± 3.17	26.44 ± 2.97
Brij® L4	150 mM	0.03 g mL^{-1}	54.50 ± 18.89	26.02 ± 4.28
Control (DMC)			38.42 ± 0.77	

The results showed a clear reduction in the amount of extracted polymer. Probably these evidences can be explained by the formation of a hydrocolloid gel that made impossible to separate the DMC phase rich in PHA from the pre treated biomass.

For this reason, in the DMC step the S/L ratio proposed by Samorì et al.[89] was kept as optimum condition, and the following extraction procedure was defined (Fig. 6.2).

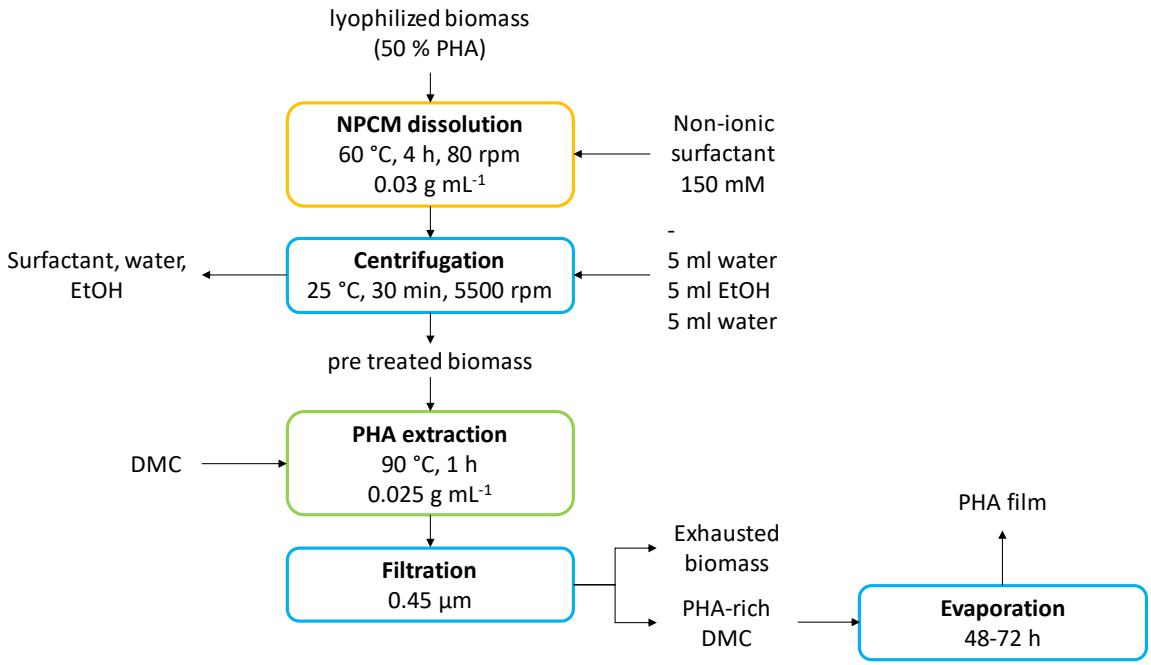

Figure 6.2 *PHAs extraction procedure developed in this work*

6.4 Conclusions

By adopting the best conditions in both the steps of the developed extraction procedure, it was possible to increase the polymer recovery yield by 50 % with respect to what obtained by performing the extraction method developed by Samorì et al.[89] on the PHA-rich mixed microbial biomass selected on fermented agro-industrial wastes.

Moreover, the best results were just a bit lower with respect to what obtained by using chloroform as PHA extractive agent, that resulted in 63 % of polymer recovery yield, with the disadvantage of the involvement of an undoubtedly toxic solvent compared to the greener alternative proposed in this study.

To complete the present work, other analyses have to be performed in order to compare the molecular weight and the purity of the extracted polymers with what reported in literature related to PHAs extraction from MMCs.

7. Conclusions

This PhD thesis was aimed to the production of polyhydroxyalkanoates by using mixed microbial cultures (MMCs) starting from several renewable and low cost feedstocks: cheese whey, the OFMSW, *Arundo donax* and CO_2.

In particular, before using these feedstocks for PHAs production, they were submitted to different acidogenic processes in order to produce liquid substrates rich in organic acids, the direct metabolic precursors of PHAs in MMCs.

All of these substrates were then used as carbon sources to successfully select MMCs with good PHAs storing ability, and for the massive biopolymer production by using the selected MMCs. The results obtained in terms of PHAs yield and PHAs content on cell dry weight were promising and comparable with the literature related to PHAs production from MMCs and complex feedstocks. In particular, during the accumulation tests, PHAs yield on substrate consumed resulted up to 0.75 ± 0.1 g COD_{PHA} g^{-1} COD by using fermented cheese whey as carbon source, 0.52 ± 0.1 g COD_{PHA} g^{-1} COD by using a percolate of the OFMSW, 0.85 ± 0.2 g COD_{PHA} g^{-1} COD by using fermented hydrolysed *Arundo donax*, and up to 1.06 ± 0.2 g COD_{PHA} g^{-1} COD by using a mixture of organic acids produced via microbial electrosynthesis (MES).

The lowest yield was detected in PHAs production from a percolate of the OFMSW, probably because of the complexity of the carbon source that acted as limiting factor during the selection of a PHAs storing MMC; in facts, in the last work performed, by using the mixture of organic acids produced via MES as substrate, the highest PHAs yield was obtained, due to the purity of the carbon source fed to the MMC that led to a selection of a MMC with high PHAs storing ability.

Depending mainly on the composition in organic acids of the substrates fed to the mixed microbial consortia, different biopolymers were produced: in particular, by using substrates rich in butyric, acetic and lactic acids (fermented cheese whey from lactic fermentation, fermented hydrolysed *Arundo donax* and the mixture of butyric and acetic acids obtained via MES) biopolymers made mainly of hydroxybutyrate (HB) were produced (HB > 96 %); while using substrates containing also propionic and valeric acids (fermented cheese whey from mixed acid fermentation and the percolate of the OFMSW), copolymers made of hydroxybutyrate and of hydroxyvalerate (HV) in different proportion were produced (namely P(HBcoHV)), 60:40

(HB:HV, %) and 55:45 (HB:HV, %), respectively for fermented cheese whey and for the percolate of the OFMSW.

In the last part of this study a novel PHAs extraction method from mixed microbial biomass was developed with encouraging results. In particular, by coupling the use of organic non-ionic surfactants to act on the non-PHA cell mass with the use of the green organic solvent dimethyl carbonate to extract PHAs, it was possible to reach biopolymer recovery yields not so far from the one obtained adopting traditional extraction methods based on the use of chloroform.

Excluding food contact, the extracted biopolymers could be used to produce packing materials, shopping bags and biodegradable mulching films to be applied in agriculture.

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